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DEVELOPING DOUBLED-HAPLOID TECHNOLOGY FOR CHICKPEA

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Developing Doubled-Haploid Technology for Chickpea

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by

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A. Abstract

The objective of this project was to develop doubled-haploid (DH) technology for chickpea and to test the developed protocol on a wide range of genotypes. An anther protocol was developed in the previous year. DH plants were produced from CDC Xena (kabuli) and Australian cultivar Sonali (desi) through a combination of stress treatments consisting of cold, centrifugation, electroporation and high osmotic stress for four days. This protocol was tested this year on five other cultivars and ten different F1 hybrids. Regeneration has been achieved for CDC Luna and Frontier, Australian cultivars Bumper and Narayen, as well as CDC hybrid 21085MM9X618. Haploid embryos have been obtained for all other genotypes and but plant regeneration is still in progress. Flow cytometric analysis of calli, embryos and regenerated plants showed haploid profiles and/or spontaneous doubling of chromosomes during early regeneration stages.

B. Executive Summary

This project involved the development of doubled-haploid technology for chickpea (*Cicer arietinum* L.) in collaboration with Dr. Janine Croser from the Centre for Legumes in Mediterranean Agriculture (CLIMA, Crawley, Australia) and Dr Sergio Ochatt from the Institut National de la Recherche Agronomique (INRA, Dijon, Cedex, France).

During the third year of this project, a major breakthrough was made in the development of a double-haploid (DH) protocol through anther culture using cultivars CDC Xena (kabuli type) and Sonali (desi type) (see Grewal et al 2009 for details). Androgenesis required the following stress treatments: (1) Flower clusters were cold treated at 4°C for 4 days, (2) Anthers were subjected to electrical shock treatment of three exponentially decaying pulses of 200 V with 25 µF and 25 Ω, (3) Anthers were then centrifuged at 168 g for 10 min. and finally, (4) Anthers were cultured for four days in high osmotic pressure (563 mmol) liquid medium namely RM-IK17. Anthers were subsequently transferred to solid embryo development medium PCL2-ppms and cultured for about four weeks until embryos were mature enough for transfer to regeneration medium . Regenerated plants from CDC Xena and Sonali showed spontaneous doubling of the chromosomes. Seeds from one CDC Xena DH_1 plant were collected and tested for viability and germination capacity. DH_2 plants grew normally and set pods. Seeds from this plant will be collected in the near future.

During the last year of this project, the developed DH protocol was tested on Australian cultivars Bumper and Narayen, CDC cultivars Luna and Frontier, ICRISAT breeding line ICCV2, and ten different hybrids from the CDC breeding program (1936as, 1936bs, 1939bs, 1946bs, 1949bs, 1990T, 2011T, 2014T, 2025T and 21085MM9X618). Haploid/DH embryos were recovered from all genotypes and plant regeneration is in various stages. In the case of Bumper, Narayen, CDC Frontier and CDC Luna, DH plants have been recovered through spontaneous doubling of chromosomes, similar to the

results obtained with CDC Xena and Sonali. DH plants of Bumper and CDC Luna are showing pod formation and seeds are maturing in the greenhouse.

Subsequent experiments were focused on improving the efficiency of the protocol and on improving regeneration rates. Modifications were made to the liquid osmotic medium used during stress treatments as well as the solid embryo development and plant regeneration media. Regeneration rates of hybrids were generally low caused by the inadequate growth conditions in the growth chambers due to infrastructure problems in the phytotron.

Further improvements of this DH protocol are still necessary to make it more cost efficient. This would involve reducing the time period required for complete DH plant production as well as increasing the regeneration rate.

C. Abbreviations

CDC	Crop Development Centre
CLIMA	Centre for Legumes in Mediterranean Agriculture, Crawley, Australia
DAFA	Department of Agriculture and Food in Western Australia
DH	Double-Haploid
INRA	Institut National de la Recherche Agronomique Dijon, France
ICRISAT	International Crop Research Institute for the Semi-Arid Tropics, Hyderabad, AP India
EDM	Embryo Development Medium
EMM	Embryo Maturation Medium
PRM	Plant Regeneration Medium
ABA	Abscisic acid
BAP	Benzyl amino purine
2,4-D	2, 4-Dichlorophenoxy acetic acid
Dicamba	3, 6-Dichloro-o-anisic acid
GA_3	Gibberellic Acid
IAA	Indole Acetic Acid
IBA	Indole Butyric Acid
2iP	6 γ-γ- Dimethyl amino purine
NAA	Naphthalene Acetic Acid
Picloram	4-Amino-3, 5, 6-trichloropiconilic acid

D. Technical Report

1. Project Background

Saskatchewan growers are estimated to have produced 100,000 tonnes of chickpea in 2009. In order to remain competitive, they continuously need improved varieties with early

maturity and higher ascochyta blight resistance. Doubled-haploid technology would speed up cultivar development for the following reasons:

- Genes are fixed in a single generation and thus the breeding process is accelerated by at least 3 years.
- The use of doubled-haploid lines in the breeding program would allow more efficient exploitation of molecular markers, which depend on homozygous populations. The length of time required to produce homozygous lines is a major limitation to the widespread adoption of molecular markers in the chickpea breeding program.
- Selection is more efficient using doubled-haploid populations than from conventional segregating breeding populations since fewer doubled-haploid plants need to be screened to cover a similar spectrum of recombinants. Early maturing, resistant cultivars would be available more quickly to farmers. This is especially important for keeping ahead of genetic changes in the *Ascochyta* blight fungus.
- Haploid microspores are excellent targets for genetic manipulation and mutagenesis studies. This is especially important in a crop such as chickpea that has very low intra-specific variability.

Doubled-haploid technology has made significant contributions to the improvement of major crops such as wheat, barley, and canola (Maluszynski *et al* 2003, Palmer *et al* 2005). To date, very little work has been done on anther culture of pulse crops (for a review see Croser *et al* 2006). A major hurdle is the recalcitrant nature of legumes to tissue culture techniques. A complete haploid legume protocol using microspore culture has only been accomplished in pigeon pea (Kaur & Bhalla 1998) but the regeneration frequency of these haploid plants ranged only from 10 to 13 per cent. There is no report of using this microspore protocol in any pigeon pea breeding program. Regeneration and conversion of embryos into plants were identified as major impediments to the development of doubled-haploid technology for chickpea as well as other legumes (Kaltchuk-Santos *et al* 1997).

Last year, a major breakthrough was made in the development of a DH protocol for chickpea via combining four different stress treatments with improved induction and regeneration media (Grewal *et al* 2009). Further changes to our protocol were needed in order to improve its efficiency and adapt it to a wide range of genotypes.

2. Project Objectives

The major objectives of this project were:

a) To develop a reproducible and efficient androgenesis protocol for chickpea
b) To produce haploid embryos
c) To convert the embryos into plantlets
d) To determine the ploidy status of the plantlets and, if necessary, to double their chromosome number
e) To root the double-haploid plants and establish them in soil

f) To screen additional genotypes available at the Crop Development Centre (Saskatoon) with the developed protocol
g) To improve the developed protocol to make it more efficient

Objectives a) to e) were completed previously. In 2009, work focused on points f) and g).

3. Results and Discussion

Developing an efficient and reproducible androgenesis protocol for chickpea is a slow and laborious process because the protocol consists of numerous steps. Each step has to function at least to some degree in order to obtain results. Evaluation of factors cannot be done in isolation since they are inter-connected. For example, in order to identify responsive genotypes, effective pre-treatments and appropriate culture media are necessary. Similarly, in order to test pre-treatments, responsive genotypes and healthy donor plants free of any kind of stress are required. Furthermore, if the culture medium and conditions are unsuitable, no induction will occur even if the genotype is responsive and the pre-treatment was effective. Hence, each step of the protocol has to be re-evaluated if other conditions are changed in order to develop a reproducible and efficient androgenesis protocol for chickpea.

3.1. Experimental Design

3.1.1. Genotypes screening and plant growth conditions

Genotype plays a predominant role in determining androgenic response (Orshinsky and Sadasivaiah 1994). Hence, a wide range of genotypes was tested using our DH protocol; specifically two CDC kabuli cultivars, CDC Luna and CDC Frontier, the Australian kabuli cultivars Bumper and Narayen (from DAFWA and CLIMA), Indian desi breeding line ICCV2 (from ICRISAT) and ten hybrids from the CDC breeding program namely 1936as, 1936bs, 1939bs, 1946bs, 1949bs, 1990T, 2011T, 2014T, 2025T and 21085MM9X618 (developed by Dr Bunyamin Tar'an).

In order to achieve high rates of androgenesis induction, it is necessary to grow the donor plants in a stress-free environment by providing optimal light intensity and spectrum, day length, temperature regime, humidity and other conditions such as a pest and disease-free environment (Dunwell and Perry 1973; Dunwell 1976).

Donor plants were raised from seed in root trainers filled with Sunshine No. 4 mix. Plants were grown in phytotron chambers (Conviron, Winnipeg, MB, Canada) at the University of Saskatchewan if available, maintained at 22/12° C (day/night) with a 17/7 h light/dark photoperiod and watered as required. Fluorescent lamps and high-intensity metal filament lamps provided light with a photosynthetic photon flux density of about 380 µmol m^{-2} s^{-1} at plant level. Two weeks after germination, seedlings were transplanted to 11 litre plastic

pots containing the same soil mix with 5 plants per pot and pots were transferred to the greenhouse.

Unfortunately, due to problems with the cooling system of the phytotron, growth chambers were run with reduced light intensity starting in late spring of 2009. This resulted in sub-optimal growth conditions and donor plant growth was negatively affected.

3.1.2. Bud size and harvest number

Successful anther culture depends upon the use of microspores at the appropriate developmental stage, usually mid- to late uni-nucleate (Falavigna 1979; Tsay et al. 1980). Our experiments showed that different genotypes contain this microspore developmental stage at different bud sizes. Buds 2- 3 mm in length were taken and the uni-nucleate microspore stage was confirmed by using DAPI staining. For desi type chickpea, the bud size that yielded anthers with mid to late uni-nucleate microspores was 2.0 to 2.8 mm; anthers at this stage had a greenish, yellow colour. For kabuli type chickpea, the bud size needed to be 2.5 to 3.0 mm to ensure the same microspore stage (data not shown). Anthers were subsequently excised for stress treatments.

Three harvests were taken at weekly intervals from each donor plant. Flower clusters containing different sized buds were picked and stored in plastic containers lined with moistened paper towels. Preliminary experiments showed that embryo formation rates were higher from re-grown flower clusters and subsequently, the first harvest of flower clusters from each donor plant was discarded and only the second and third collections were used for experiments.

3.1.3. Stress treatments

Once the donor plant material has been selected, development of the microspores has to be switched from the gametophytic to the sporophytic pathway. Pre-treatments can be applied at different stages and to different parts of the donor plant, for example whole plants or plant parts such as clusters of buds, single flower buds, anthers or isolated microspores. Specific stress pre-treatments are required such as cold, centrifugation (Tanaka, 1973), electric or osmotic shock (Delaitre *et al.* 2001 and Ochatt *et al.* 2007, 2009); treatments were applied alone or in combination.

The following stress treatments were applied to cultivars and breeding lines:

- Cold (CL) treatment of flower clusters was applied to all plant material used in this study
- Cold treatment of flower clusters followed by culturing anthers in high osmotic shock (O) medium for 4 days prior to the transfer to solid medium

- ❖ Cold treatment of flower clusters followed by centrifugation (C) of anthers at 168 g for 10 min and osmotic shock for 4 days prior to the transfer to solid medium

- ❖ Cold treatment of flower clusters followed by electroporation (E) of anthers using 200 Volts and osmotic shock for 4 days prior to the transfer to solid medium

- ❖ Cold treatment of flower clusters followed by centrifugal, electrical and osmotic shock of anthers

After applying these stress treatments, anthers were cultured on different solid embryo development media (EDM).

3.1.4. Anther culture

After the different pre-treatments anthers were cultured on either the same solid embryo development medium (EDM) used in the developed protocol or a modified version of this medium (Phillips and Collins 1979, PC-L2, Appendix 2) using 14x58 mm Star™ dish plates (Phoenix Biomedical, Mississauga ON Canada). For high osmotic pressure stress, anthers were induced in different liquid media for four days prior to transfer to solid embryo development medium. Anthers from cold-treated flower clusters (CL) served as control. Each experiment had three replications with 30 anthers per plate. Cultures were incubated at 24 ± 2° C in darkness in an incubator (Fisher Scientific low temperature incubator, Pittsburgh PA, USA). Beginning 15 days after culture initiation, data was collected on percent anther induction as well as number of embryos formed. The anther induction rate was determined using the following equation:

$$\text{Anther Induction (\%)} = \frac{\text{Number of anthers producing callus}}{\text{Total number of anthers cultured}} \times 100$$

Callus or anthers that exhibited embryo formation were transferred to a solid embryo maturation medium (EMM; Appendix 3) and maintained as above.

Mature embryos (cotyledonary phase) were transferred to plant regeneration medium (PRM, Appendix 4) and maintained at 23° C using a 16/8 h light/dark photoperiod in a Sanyo versatile environmental test chamber (Concord, ON, Canada). Fluorescent tubes provided light with a photosynthetic photon flux density of 45 µmol m^{-2} sec^{-1}. Green plants were transferred to soil and grown in a growth chamber as described previously. After four weeks, they were moved to the greenhouse until seed harvest.

The ploidy level of calli, embryos and regenerated plants was determined by using a Partec Flow Cytometer (CCA New Jersey, USA). Flow Cytometer facility was provided by Dr. Alison Ferrie from the Plant Biotechnology Institute, National Research Council of Canada (PBI-NRC, 110 Gymnasium Place, Saskatoon SK). Leaf material (1 - 2 mm) from CDC Xena and Sonali plants grown from seed (control) as well as calli, embryos and plants regenerated through anther culture were chopped in 400 µl nuclei extraction

buffer (Partec®) followed by the addition of 1.6 - 2.0 ml staining buffer (Partec®). The chopped material was sieved through 30 µm cell-trics from Partec® and the filtered suspension was run through the flow cytometer for DNA profiles. Analysis of variance was calculated using PROC GLM (General Linear Model) of SAS 9.2 (SAS Institute Inc. 2009). Least significant differences (LSD) were calculated (P = 0.05) for each treatment for comparison of means.

3.1.5. Embryo development and plant regeneration

After 3-5 weeks of culture anthers showed induction and embryo formation. Several different types of embryos were obtained in our experiments: smooth surface globular, rough surface, irregular shaped, or cotyledonary embryos. These embryos were further transferred to embryo maturation medium (EMM, Appendix 3).

Mature embryos were subsequently transferred from EMM to different regeneration media (Appendix 4) and subsequently, six types of developments were observed:

- Differentiation into complete plantlets with root and shoot
- Differentiation into shoots
- Differentiation into roots
- Differentiation into green islets
- Differentiation into green islets with roots
- No differentiation of embryos

Experiments showed that plantlets resulting from electroporated anthers had improved root induction and development, similar to that of CDC Xena and Sonali. Addition of calcium pantothenate to the regeneration medium was found to aid embryo conversion and plantlet growth. Data on plantlet, shoot, and root formation is presented for the following cultivars.

3.2. Cultivars and breeding lines

3.2.1. Cultivar Bumper

Bumper is an Australian cultivar of the kabuli type. This cultivar was found responsive to the developed DH protocol and anthers were induced in nine different osmotic media (for composition see Appendix 1) used during stress treatments and then cultured on different solid embryo development media (EDM, Appendix 2) for embryo induction. Table 1 presents the data obtained from the different experiments; treatments that resulted in the highest embryo numbers are highlighted in bold. Percent anther induction rates ranged between 0 to 93% with the highest rate resulting from osmotic shock in RM-IK2NF medium (Appendix 1) for 4 days prior to the transfer to D2 medium (Appendix 2).

The best treatment for embryo production in this cultivar was the combination of all the stresses (CL-CEO) using the ChM-GM medium (Appendix 1) and PCL2-ppms medium

Table 1. Effect of different stress treatments, liquid and/or solid media on anther and embryo induction for cultivar Bumper

Stress Treatment CL	C	E	O	Osmotic Medium	EDM	Incubation Temp ° C	Hours of incuba-tion	% Anther induction	Embryo No
+	-	-	-		PCL2-ppms	24		43	37
+	-	-	-		PCL2-ppmsA	24		34	11
+	-	-	-		PCL2-ppms	28		3	0
+	-	-	-		CIM-ppms	24		22	4
+	-	-	+	JC15	PCL2-ppms	28	24	63	4
+	-	-	+	JC15	PCL2-ppmsA	28	24	65	32
+	-	-	+	JC15	CIM-ppms	28	24	50	4
+	+	-	+	RM-IK17	CIM-ppms	24		77	7
+	-	+	+	RM-IK17	CIM-ppms	24		27	1
+	+	+	+	RM-IK17	CIM-ppms	24		57	5
+	+	-	+	RM-IK17SP	CIM-ppms	24		46	10
+	+	-	+	RM-IK17SP	CIM-ppms	28	24	50	5
+	+	-	+	RM-IK17SP	PCL2-ppms	24		0	0
+	+	-	+	RM-IK17SP	PCL2-ppms	28	24	23	0
+	+	-	+	RM-IK9	PCL2-ppms	24		57	7
+	+	+	+	RM-IK9	PCL2-ppms	24		28	1
+	-	-	+	RM-IK17	PCL2-ppms	24		35	24
+	+	-	+	RM-IK17	PCL2-ppms	24		47	40
+	-	+	+	RM-IK17	PCL2-ppms	24		61	30
+	+	+	+	RM-IK17	PCL2-ppms	24		49	37
+	-	-	+	RM-IK17SP	PCL2-ppmsA	24		10	0

+	-	-	+	RM-IK17SP	PCL2-ppmsA	28	24	10	0
+	-	-	+	RM-IK17pH5.9	PCL2-pps	24		50	7
+	+	+	+	RM-IK17pH5.9	PCL2-pps	24		2	40
+	+	+	+	RM-IK17pH7.0	PCL2-pps	24		40	0
+	-	-	+	RM-IK2F	PCL2-ppms	24		58	37
+	+	-	+	RM-IK2F	PCL2-ppms	24		78	79
+	-	+	+	RM-IK2F	PCL2-ppms	24		90	87
+	+	+	+	RM-IK2F	PCL2-ppms	24		87	65
+	-	-	+	RM-IK2F	PCL2-ppmss	24		45	21
+	-	-	+	RM-IK2NF	PCL2-ppms	24		81	68
+	+	-	+	RM-IK2NF	PCL2-ppms	24		80	84
+	+	-	+	RM-IK2NF	PCL2-ppmsA	24		43	3
+	-	+	+	RM-IK2NF	PCL2-ppms	24		80	85
+	+	+	+	RM-IK2NF	PCL2-ppms	24		90	60
+	-	-	+	RM-IK2NF	D2	24		93	5
+	-	-	+	RM-IK17	PCL2-ppms	28	24	70	79
+	-	-	+	RM-IK17	PCL2-ppms	32.5	16	72	113
+	-	-	+	RM-IK17SP	PCL2-ppmss	24		53	81
+	-	-	+	RM-IK17SP	PCL2-ppmss	32.5	16	67	110
+	-	-	+	RM-IK17	D3	24		52	10
+	-	-	+	RM-IK17	D3	32.5	16	52	3
+	-	-	+	ChM-GM	PCL2-	24		78	114

					ppms				

Legend:
CL = Cold treated flower clusters (control)
C = Centrifugation using 168g for 10 min.
E = Electroporation using 3 pulses with 200 V, 25 Ω and 25 µF
O = Osmotic shock, keeping anthers in high osmotic pressure medium for 4 days prior to transfer to embryo development medium
EDM = Embryo Development Medium
LSD = Least significant difference at 0.05

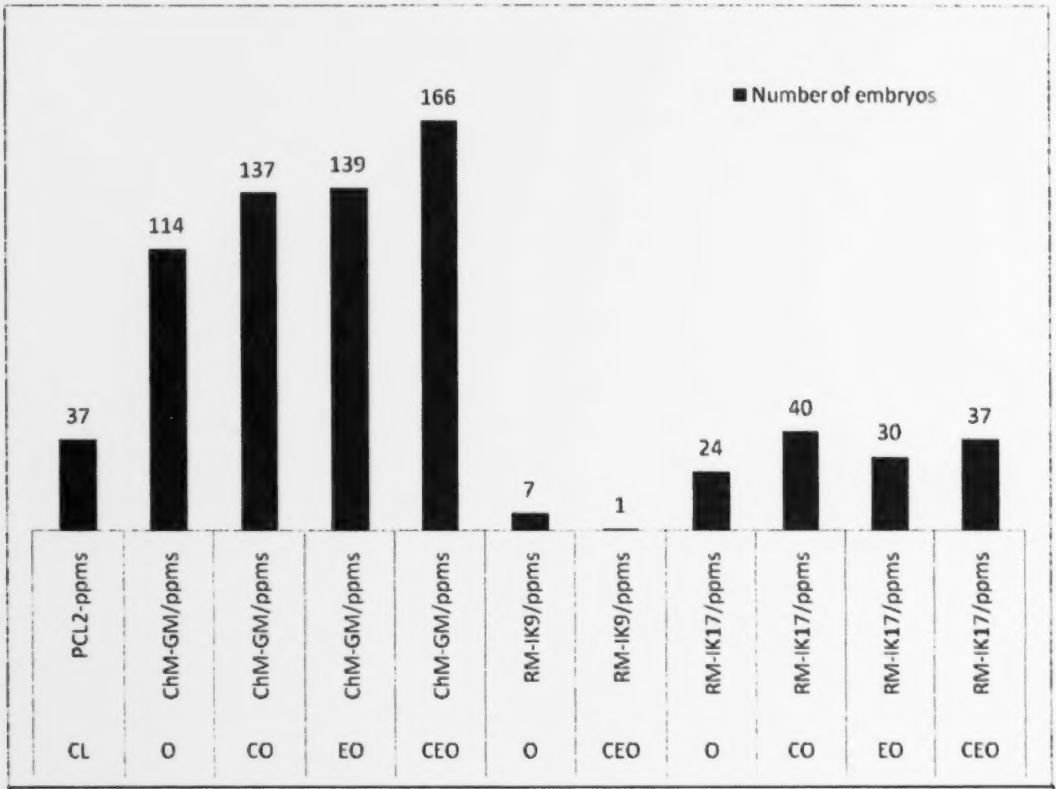
+	+	-	+	PCL2-ppmSP	PCL2-ppmss	24		53	45
+	+	+	+	PCL2-ppmSP	PCL2-ppmss	24		50	46
							LSD	**16.8**	**11.4**

(Appendix 2). This treatment produced 166 embryos (Fig. 1). Using the same medium combination, electroporated anthers (CL-EO) produced 139 embryos, centrifuged anthers (CL-CO) produced 137 embryos and osmotic shock (CL-O) produced 114 embryos. All these treatments in combination with this medium produced significantly higher number of embryos at P>0.05 (Table 1). However, even though the frequency of embryo formation was higher than any other treatment, the embryos could not be regenerated into plantlets.

Cistué *et al* (2009) reported the beneficial effect of adding Ficoll-400 in the culture medium for DH production in durum wheat. Ficoll-400 is a non-ionic synthetic polymer of sucrose used for cell separation and organelle isolation. Embryos develop much better in medium with high osmotic pressure or high viscosity, which was translated into an increased number of dividing microspore. Similar results were obtained by Immonen and Robinson (2000) for increased green plant regeneration with triticale. Kao (1981) used Ficoll-400 in barley anther culture in order to prevent microspore submersion and anaerobic conditions, thereby promoting embryo normal development. We also studied the effect of Ficoll (F) on embryo induction by modifying the osmotic shock medium (RM-IK) with 300 g/l Ficoll-400 (Fig. 2). Results of this study showed that the addition of FIcoll-400 had no beneficial effects for embryo production when anthers were subjected to electroporation since the number of embryos was 87 in RM-IK-2F medium (Fig. 2) and 85 embryos in medium 9RM-IK2NF without Ficoll-400 (Table1) and this difference was not significant.

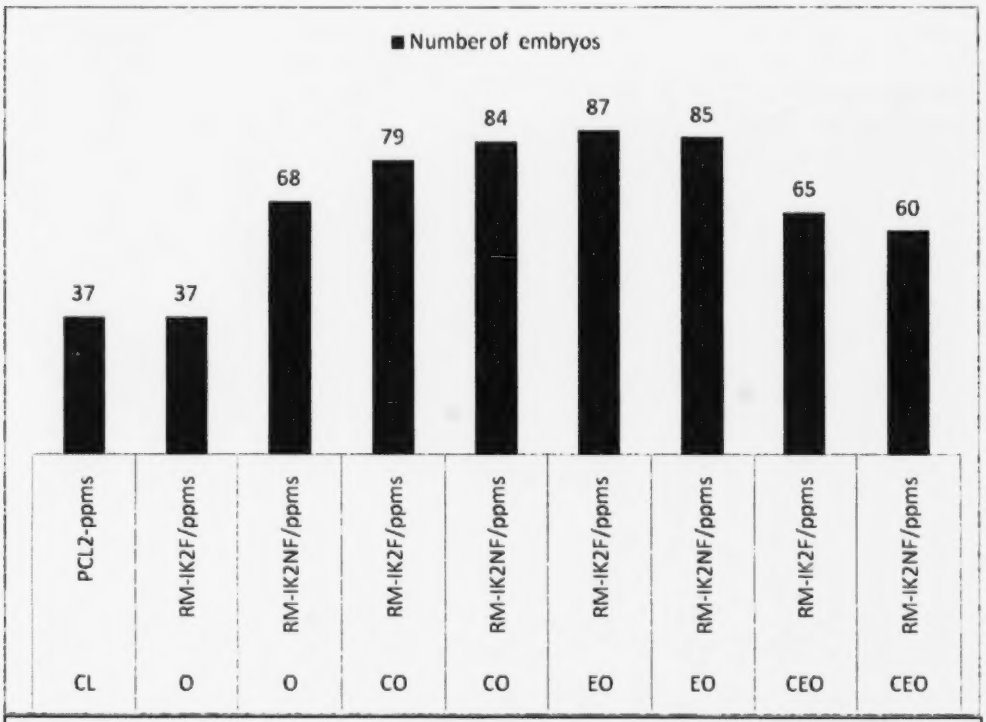
Two other treatment combinations also showed high frequency of embryo formation; the first one was osmotic shock (CL-O) of anthers in RM-IK17 prior to transfer to PCL2-ppms which produced 113 embryos (anthers kept at 32.5° C for 16 hours then moved to 24° C) and the second one was osmotic shock (CL-O) in RM-IK17SP prior to transfer to PCL2-ppmss medium which produced 110 embryos (anthers kept at 32.5° C for 16 hours and then moved to 24° C) (Appendix 1 & 2).

Fig. 1. Effect of stress treatments and various osmotic shock media on embryo Production for cultivar Bumper



Legend:
PCL2-ppms = Embryo Development medium (EDM)
ChM-GM/ppms = ChM-GM (osmotic medium) / PCL2-ppms (EDM)
RM-IK17 / ppms = RM-IK17 (osmotic medium) / PCL2-ppms (EDM)
CL = cold control
C = Centrifugation using 168 g for 10 min.
E = Electroporation using 3 pulses with 200 V, 25Ω and 25 µF
O = Osmotic shock for 4 days

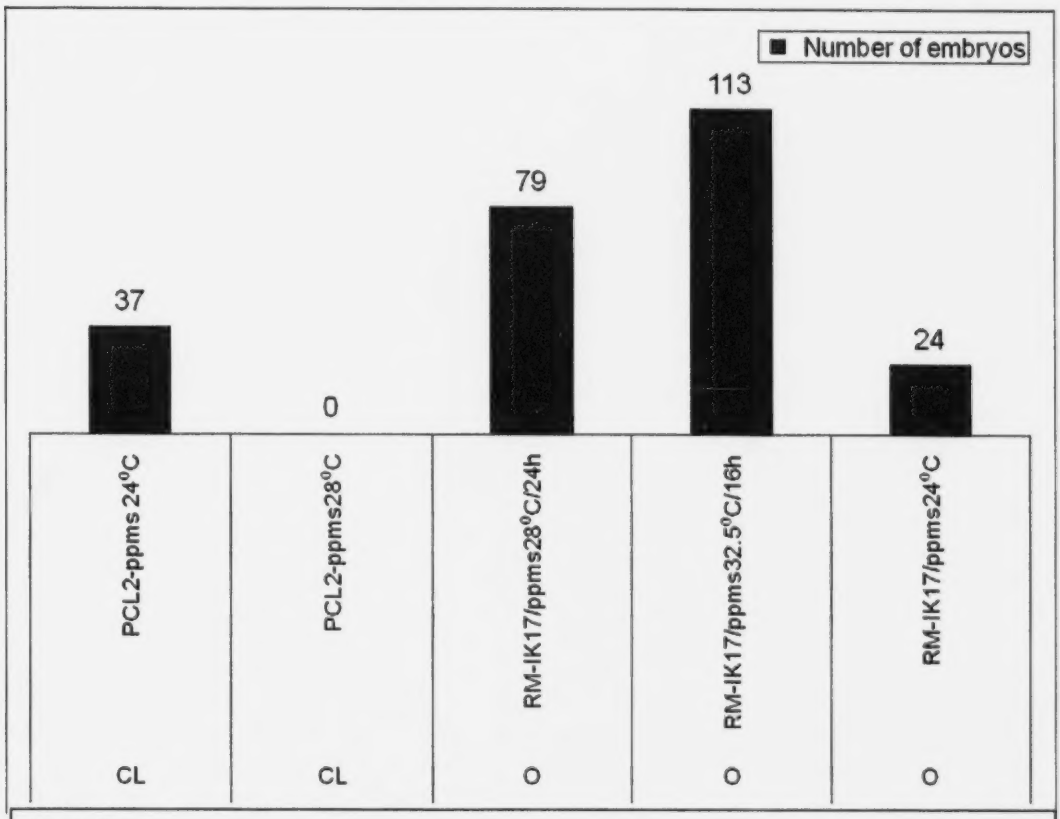
Fig. 2 Effect of Ficoll addition during high osmotic stress treatment on embryo production



Legend:
PCL2-ppms = Embryo Development medium (EDM)
RM-IK2F/ppms = RM-IK2F (osmotic medium with Ficoll-400) / PCL2-ppms (EDM)
RM-IK2NF/ppms = RM-IK2NF (osmotic medium, no Ficoll-400) / PCL2-ppms (EDM)
CL = cold control
C = Centrifugation at 168g for 10 min.
E = Electroporation using 3 pulses of 200 V for 25Ω and 25μF
O = Osmotic shock for 4 days

Figure 3 shows the effect of different incubation temperatures during osmotic shock treatment on embryo development. Anthers were cultured in RM-IK17 for four days and kept at 28 °C for 24 hours or 32.5 °C for 16 hours. Anthers kept at 24°C served as control. Anthers incubated at 32.5°C for 16 hours produced significantly more embryos (113) compared to 28°C (79 embryos) or 24°C (24 embryos; Table 1). Combining heat shock with high osmotic medium was clearly beneficial for cultivar Bumper.

Fig. 3 Effect of different incubation temperatures during osmotic shock treatment on embryo development

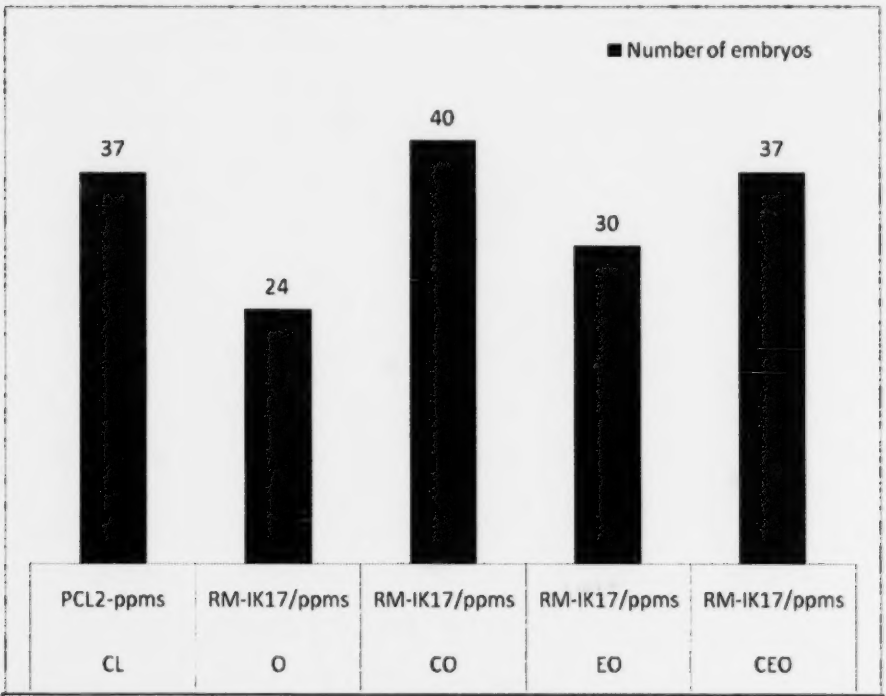


Legend:
RM-IK17 / ppms = RM-IK17 (osmotic medium) / PCL2-ppms (EDM)
PCL2-ppms 24°C = Anthers on EDM incubated at 24°C
PCL2-ppms 28°C = Anthers on EDM incubated at 28°C
RM-IK17/ppms 28°C / 24 = Anthers induced in RM-IK17 (osmotic medium) and incubated at 28°C for 24 hours and then transferred to ppms (PCL2-ppms, EDM)
RM-IK17/ppms 32.5°C / 16 = Anthers induced in RM-IK17 (Osmotic medium) and incubated at 32.5°C for 16 hours and transferred to ppms (PCL2-ppms, EDM)
CL = Cold treatment (control)
C = Centrifugation at 168g for 10 min.
E = Electroporation using 3 pulses of 200 V for 25Ω and 25μF
O = Osmotic shock for 4 days

A comparison of different stress treatments was studied using RM-IK 17 as an osmotic medium and PCL2-ppms as embryo development medium. Results showed that the

combination of centrifugation and osmotic shock (C+O) treatment was more effective than any other treatment resulting in the production of 40 embryos (Fig. 4). A visual comparison of some these treatments on anther and embryo induction are presented in the photo file, Fig. 6.

Fig. 4 Effect of stress treatments on embryo production

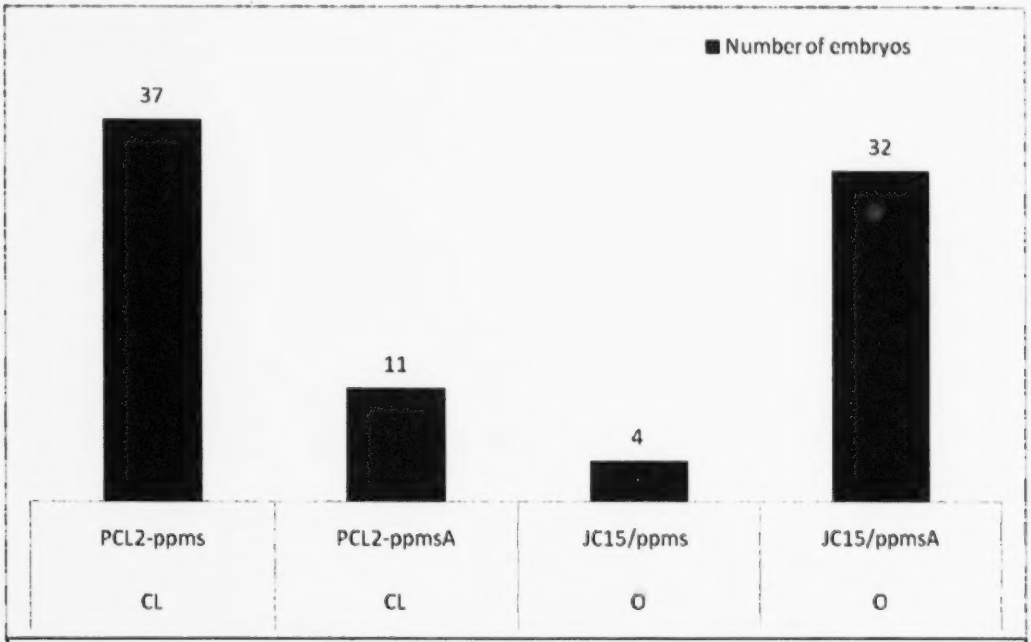


Legend:
PCL2-ppms = Embryo Development medium (EDM)
RM-IK17 / ppms = RM-IK17 (osmotic medium) / PCL2-ppms (EDM)
CL = Cold treatment (control)
C = Centrifugation at 168g for 10 min.
E = Electroporation using 3 pulses of 200 V for 25Ω and 25μF
O = Osmotic shock for 4 days

The following experiment tested if the medium sterilization method (filter-sterilizing or autoclaving) had an effect on embryo formation rates using PCL2-ppms. The filter-sterilization method consisted of all medium components except the agar being filtered through a 0.2 μm bottle top filter, the agar being autoclaved at 15 psi (120° C) for 30 min and then both components being mixed together prior to pouring the plates (PCL2-ppms; Appendix 2). The autoclaving method consisted of all components of the medium including the agar being autoclaved together at 15 psi for 30 min (PCL2-ppmsA; Appendix 2). Autoclaving affects the hydrolysation of

carbohydrates such as sucrose and maltose into its components glucose and fructose and thus also changes the osmotic strength of the medium.
Figure 5 shows that when using only cold as a stress treatment (i.e. control; no high osmotic medium required) 37 embryos were produced on filter-sterilized medium PCL2-ppms whereas only 11 embryos developed on autoclaved medium. In contrast, the opposite result was obtained when the combination cold and osmotic shock (JC15 used during osmotic shock) was used as a stress treatment. In this case the autoclaved PLC2-ppms medium resulted in more embryos (32) compared to the filter-sterilized medium (4 embryos). Differences among these treatments were also statistically significant at P>0.05 (Table 1).

Fig. 5 Effect of medium sterilization method on embryo production



Legend:
PCL2-ppms = EDM filter-sterilized
PCL2-ppmsA = EDM sterilized by autoclaving
JC15 / ppms = JC15 (osmotic medium) / PCL2-ppms = EDM filter-sterilized
JC15 / ppmsA = JC15 (osmotic medium) / PCL2-ppms = EDM sterilized by autoclaving
CL = Cold treatment (control)
C = Centrifugation at 168g for 10 min.
E = Electroporation using 3 pulses of 200 V for 25Ω and 25μF
O = Osmotic shock for 4 days

After induction embryos were transferred to medium EMM-B (see Appendix 3) for maturation. Mature embryos of cultivar Bumper (see Photo file Fig. 7A) were then

transferred to plant regeneration medium PRM-JR1 (see Appendix 4) for plantlet formation (see Photo file Fig. 8A).
For cultivar Bumper, data on percent anther induction, number of embryos produced and regeneration obtained from all the osmotic and embryo development medium combinations is presented in Table 2. In total, seven plantlets were regenerated and transferred to soil but only one plant survived due to mould contamination in the growth chamber. This plant has shown pod formation (see Photo file Fig. 10A) and seeds will be harvested in the near future. In contrast, even though embryo numbers were very high in the medium ChM-GM and PCL2-ppms combination (see Table 1 combined treatment CL-CEO), these embryos did not regenerate into plants but only into roots (see Photo file Fig. 9B) indicating auxin abnormalities in the embryos. Future experiments will need to focus on either reducing the IAA content of the osmotic medium or the length of exposure to this medium.

Table 2. Effect of stress treatments on anther induction, embryo formation and plant regeneration of Bumper

Treatments	% of anthers showing induction	Number of embryos produced	No. of embryos that formed Plants (P) Shoots (S) Roots (R)	No. of plants that survived in soil
Cold control	26	17	0	0
Osmotic shock	53	37	0	0
Centrifugation + Osmotic shock	53	37	1(P)	1(Fig.10A)
Electroporation + Osmotic shock	65	65	2(P) 3(R)	0
Centrifugation + Electroporation + Osmotic shock	58	45	4(P) 13(R)	0

3.2.2. Cultivar CDC Luna

CDC Luna (kabuli) was developed by the Pulse Breeding Program at the Crop Development Centre, University of Saskatchewan, Saskatoon. Anther induction ranged from 0 to 92 % whereas embryo numbers varied between 0 and 254 (Table 3). Maximum anther induction rates were observed in two treatments:

a) When anthers were submitted to cold and osmotic shock treatment (CL-O) in RM-IK-2NF medium (Appendix 1) for 4 days prior to transfer to PCL2-ppmsA medium (Appendix 2; 'A' denotes that this medium was sterilized by autoclaving)
b) In HSOP medium combined with stresses cold, centrifugation, electroporation and osmotic shock (CL-CEO) in HSOP prior to transfer to PCL2-ppmss medium (Appendix 1 & 2).

In contrast, the highest embryo number of 254 was observed from the cold, centrifuged and osmotic shock (CL-CO) in HSOP medium treatment and subsequent transfer of anthers to PCL2-ppmss (see Appendix 1 & 2). The second best treatment was via only cold and osmotic shock (CL-O) using the same medium combination resulting in 178 embryos (Table 3).

Table 3. Effect of different stress treatments, liquid and/or solid medium on anther and embryo induction for cultivar CDC Luna

Treatment				High Osmotic Medium	Embryo Development Medium	% Anther induction	Number of embryos
CL	C	E	O				
+	-	-	-	-	PCL2-pps	33	33
+	-	-	-	-	PCL2-ppms	3	3
+	-	-	-	-	PCL2-ppmss	76	76
+	-	-	-	-	PCL2-ppmsl	56	56
+	-	-	+	RM-IK17	PCL2-pps	46	46
+	+	-	+	RM-IK17	PCL2-pps	19	8
+	+	+	+	RM-IK17	PCL2-pps	29	31
+	-	-	+	RM-IKpH5.9	PCL2-pps	39	1
+	+	-	+	RM-IKpH5.9	PCL2-pps	39	5
+	-	+	+	RM-IKpH5.9	PCL2-pps	29	5
+	+	+	+	RM-IKpH5.9	PCL2-pps	30	6
+	-	+	+	RM-IKpH5.9	CIM-ppms	15	2
+	-	-	+	RM-IKpH7.0	PCL2-pps	29	1
+	+	-	+	RM-IKpH7.0	PCL2-pps	28	9
+	-	+	+	RM-IKpH7.0	PCL2-pps	40	5
+	+	+	+	RM-IKpH7.0	PCL2-pps	43	9
+	-	-	+	RM-IK17	D1	12	0
+	+	+	+	RM-IK17	D1	0	0
+	-	-	+	RM-IK17	D2	16	0
+	+	+	+	RM-IK17	D2	0	0
+	-	-	+	RM-IK17	D3	23	0
+	-	-	+	RM-IK17	D4	13	68
+	-	-	+	RM-IK17	D5	23	0
+	-	-	+	RM-IK17	D6	13	2
+	-	-	+	RM-IK17	D8	13	0
+	+	+	+	RM-IK17	D8	32	4
+	-	-	+	RM-IK17	PCL2-ppms	10	0
+	+	-	+	RM-IK17	PCL2-ppms	9	4
+	-	+	+	RM-IK17	PCL2-ppms	14	9
+	+	+	+	RM-IK17	PCL2-ppms	13	10
+	-	-	+	RM-IK2F	PCL2-ppmsA	21	11
+	+	-	+	RM-IK2F	PCL2-ppmsA	48	63

+	-	-	+	RM-IK2NF	PCL2-ppmsA	92	48

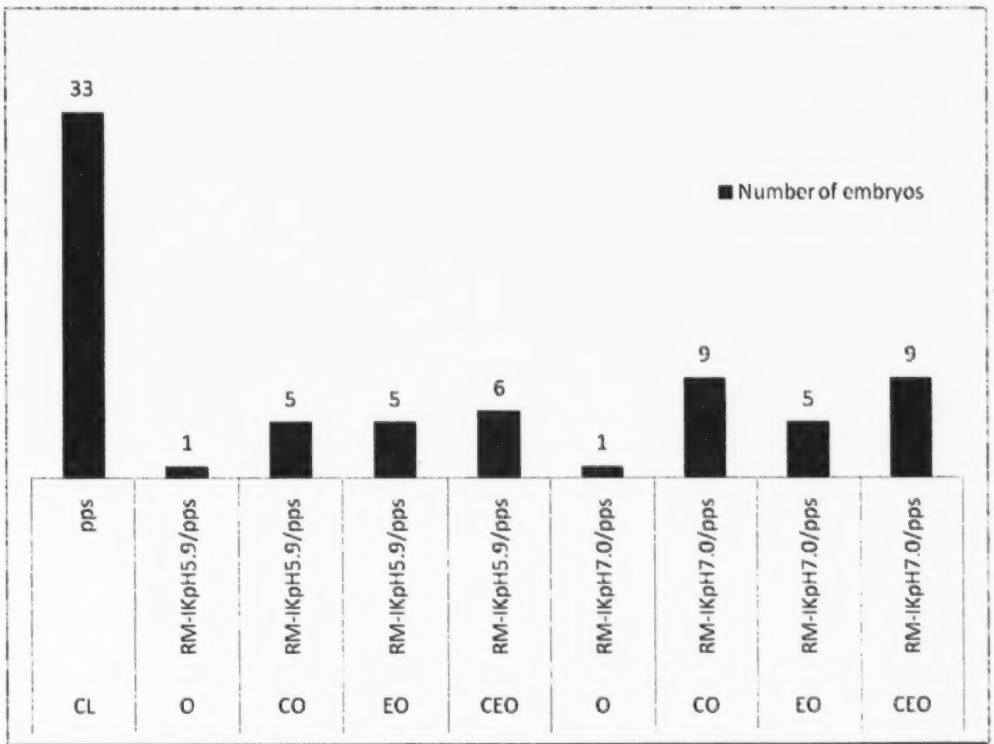
Legend:
CL = Cold treated flower clusters (control)
C = Centrifugation stress treatment at 168 g for 10 min.
E = Electroporation with 3 pulses using 200 V, 25 Ω and 25 µF
O = Anthers kept in high osmotic pressure medium for 4 days prior to transfer to EDM
LSD = Least significant difference at 0.05

+	-	-	+	RM-IK17	PCL2-ppmss	20	6
+	-	+	+	RM-IK17	PCL2-ppmss	50	0
+	+	+	+	RM-IK17	PCL2-ppmss	92	4
+	-	-	+	RM-IK2F	PCL2-ppmss	82	79
+	+	-	+	RM-IK2F	PCL2-ppmss	18	4
+	-	+	+	RM-IK2F	PCL2-ppmss	43	22
+	+	+	+	RM-IK2F	PCL2-ppmss	13	3
+	-	-	+	RM-IK2NF	PCL2-ppmss	69	25
+	+	-	+	RM-IK2NF	PCL2-ppmss	17	3
+	+	+	+	RM-IK2NF	PCL2-ppmss	8	3
+	-	-	+	RM-IK17SF	PCL2-ppmsl	41	35
+	+	-	+	RM-IK17SF	PCL2-ppmsl	5	29
+	-	-	+	RM-IK1	PCL2-ppmsl	41	28
+	-	+	+	RM-IK1 to RM-IK17SF	PCL2-ppmsl	50	57
+	+	+	+	RM-IK1 to RM-IK17SF	PCL2-ppmsl	20	4
+	-	-	+	RM-IK17 to RM-IK17SF	PCL2-ppmsl	28	26
+	+	-	+	RM-IK17 to RM-IK17SF	PCL2-ppmsl	18	9
+	-	+	+	RM-IK17 to RM-IK17SF	PCL2-ppmsl	31	41
+	+	+	+	RM-IK17 to RM-IK17SF	PCL2-ppmsl	21	11
+	+	-	+	RM-IK17	PCL2-ppmsl	9	0
+	-	+	+	RM-IK17	PCL2-ppmsl	14	6
+	+	+	+	RM-IK17	PCL2-ppmsl	13	7
+	-	+	+	RM-IK1	PCL2-ppmsl	38	37
+	+	+	+	RM-IK1	PCL2-ppmsl	29	11
+	+	+	+	HSOP3	PCL2-ppms	75	45
+	+	+	+	HSOP3	PCL2-ppmsA	14	4
+	+	+	+	HSOP3	CIM-ppms	17	2
LSD 0.05						**12.6**	**10.2**

Manipulation of the pH of the induction medium are a novel approach (Barinova *et al.* 2004) and a high pH of 8.0 to 8.5 for 4 to 6 days has resulted in symmetrical nuclei divisions of tobacco and snapdragon microspores. The divisions occurred at significantly higher frequencies producing two large nuclei of equal size. Following the initial incubation at high pH upon transfer of these microspores to a medium with a pH of 6.5, multi-cellular structures were obtained in both species.

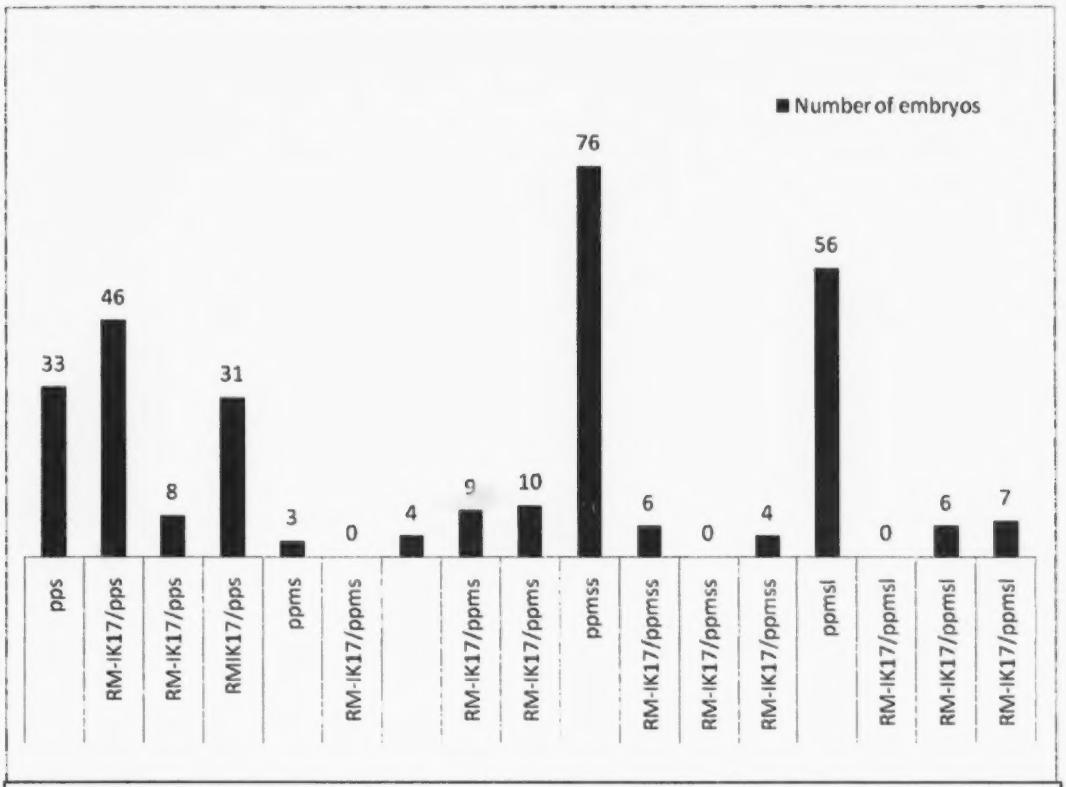
For chickpea, the RM-IK medium pH was adjusted to 5.9 or 7.0; anthers were given the usual stress treatments (OS, CO, EO, CEO) and then cultured on PCL2-pps medium (Appendix 1 & 2). Anthers cultured on PCL2-pps medium served as control. In this experiment medium pH failed to show any improvement in embryo production as compared to the control (Fig.11). Control anthers produced a maximum of 33 embryos as compared to 9 embryos from the medium with a pH of 7.0; this difference was statistically significant at P>0.05 (Table 3).

Fig.11. Effect of osmotic shock medium pH and other stress treatments on embryo production of CDC Luna



Legend:
pps = PCL2-pps (EDM)
RM-IKpH 5.9/pps = RM-IKpH5.9 (osmotic medium at pH 5.9) / PCL2-pps (EDM)
RM-IKpH7.0/pps = RM-IKpH7.0 (osmotic medium at pH7.0) / PCL2-pps (EDM)
CL = Cold treatment (control)
C = Centrifugation at 168 g for 10 min.
E = Electroporation using 3 pulses of 200 V for 25Ω and 25μF
O = Osmotic shock for 4 days

Fig.12. Effect of stress treatments and combination of osmotic medium with different solid media on embryo production of CDC Luna



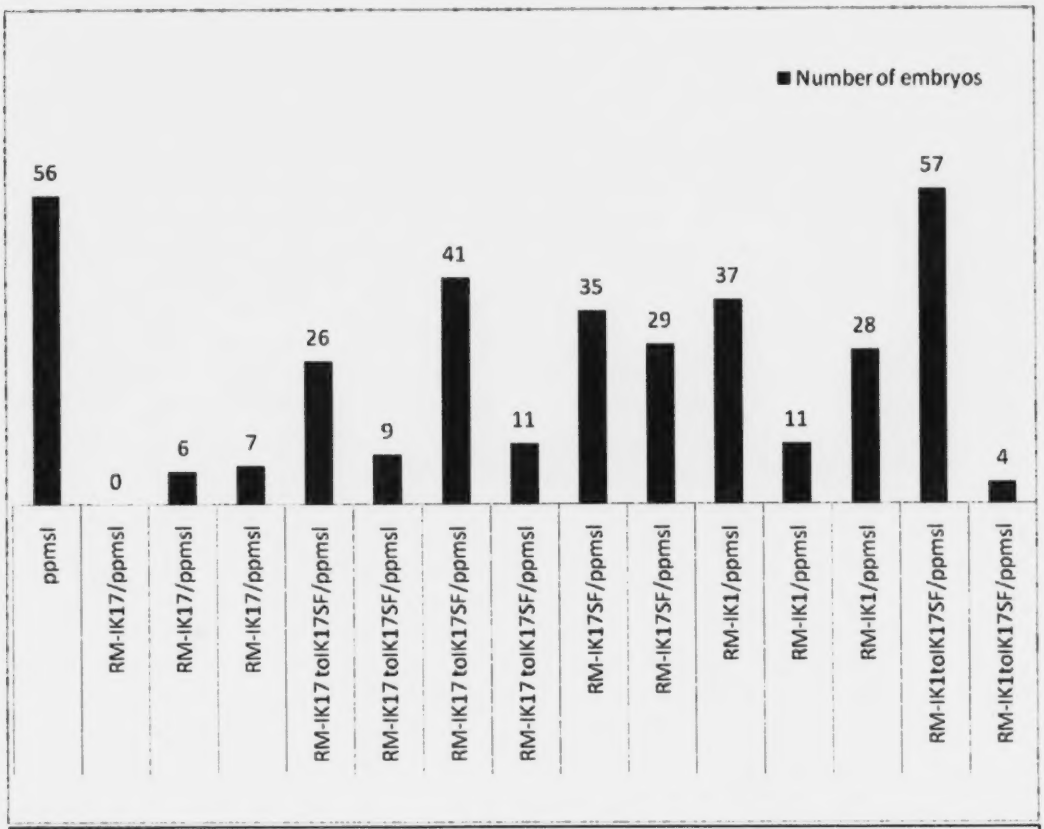
Legend:
pps = PCL2-pps (EDM)
ppms = PCL2-ppms (EDM)
ppmss = PCL2-ppmss (EDM)
ppmsl = PCL2-ppmsl (EDM)
RM-IK17/ pps = RM-IK17 (osmotic medium) / PCL2-pps (EDM)
RM-IK17/ ppms = RM-IK17 (osmotic medium) / PCL2-ppms (EDM)
RM-IK17/ ppmss = RM-IK17 (osmotic medium) / PCL2-ppmss (EDM)
RM-IK17/ ppmsl = RM-IK17 (osmotic medium) / PCL2-ppmsl (EDM)
CL = Cold treatment (control)
C = Centrifugation at 168g for 10 min.
E = Electroporation using 3 pulses of 200 V, 25 Ω and 25 µF
O = Osmotic shock

To improve the frequency of embryo formation, modifications to the PCL2-ppms medium were tested. The ratio of the two sugars (3% sucrose and 6% maltose) used in the PC-L2 medium was changed to 6% sucrose and 3% maltose (PCL2-ppmss) and the addition of larcoll (PCL2-ppmsl; Appendix 2). A comparison of four solid media (PCL2-pps, PCL2-ppms, PCL2-ppmss and PCL2-ppmsl) for embryo production was also studied; the osmotic medium was kept constant in each treatment. Anthers cultured directly on these media after cold flower cluster treatment (CL) served as control. Results are presented in Table 12. The maximum frequency of 76 embryos was observed in cold treated anthers cultured on the 6% sucrose + 3% maltose PCL2-ppmss medium, followed by 56 embryos in cold treated anthers on PCL2-ppmsl (with larcoll) medium (Fig.12). However, these media did not improve embryo production in anthers treated with centrifugation, electroporation or osmotic shock (O, CO, EO, CEO) and the differences were statistically significant at P >0.05 (Table 3). Medium PCL2-pps produced 46 embryos in the osmotic shock treatment of anthers, followed by cold treatment of clusters (33 embryos) and then by the CEO treatment (31 embryos, Fig. 12).

An experiment was conducted to study the effect of adding 300 g/l Ficoll-400 (RM-IK17SF; Appendix 1) during the 4-day osmotic shock treatment. These anthers were then moved to the solid PCL2-ppmsl medium (Appendix 2). Since the Ficoll medium has very low conductivity and high viscosity, RM-IK17S or RM-IK1 medium were used during the other stress treatments (CO, EO or CEO). Anthers cultured directly on PCL2-ppmsl medium served as control (CL). Results presented in Fig. 13 show that anther electroporation in RM-IK1 followed by osmotic shock in RM-IK17SF produced 57 embryos compared to 56 embryos produced in the control treatment. This difference was not statistically significant at P> 0.05 (Table 3).

For CDC Luna, data on percent anther induction, number of embryos produced and plant regeneration for all medium combinations is presented in Table 4. Just using cold as a pre-treatment produced more embryos than any other stress but embryos could not be regenerated into plants. Using the combination electroporation and osmotic shock (E+O) or centrifugation/electroporation/osmotic shock (CEO) resulted in fewer embryos but some were regenerated into plants (Table 4). Figure 7B (Photo file) shows embryo formation from the combined CEO stress treatment with RM-IK17 used during osmotic shock and PCL2-pps medium for embryo development (Appendix 1 & 2). These embryos were transferred to embryo maturation medium EMM-1 (Appendix 3) for further maturation. The mature embryos of this cultivar were then transferred to plant regeneration medium JR-1 (Appendix 4) for plantlet formation. Fig. 8B (Photo file) shows the DH plantlet regenerated from CDC Luna. A total five plantlets and eight shoots (no roots) were obtained (Table 4). Rooted plantlets were transferred directly to soil but only one survived. This plant has produced pods (Photo file Fig. 10B). Shoots were moved to filter-paper bridges using a liquid JR-1-1 regeneration medium with hormones for root formation (Appendix 4) but roots did not develop.

Fig.13. Effect of Ficoll-400 during osmotic shock treatment on embryo production



Legend:
ppmsl = PCL2-ppmsl (EDM)
RM-IK17/ppmsl = RM-IK17 (osmotic medium) / PCL2-ppmsl (EDM)
RM-IK17SF/ppmsl = RM-IK17SF (osmotic medium with Ficoll-400) / PCL2-ppmsl (EDM)
RM-IK17 /ppmsl = RM-IK1 (osmotic medium) / PCL2-ppmsl (EDM)
RM-IK17 to RM-IK17SF / ppmsl = Anthers induced in RM-IK17 (osmotic medium) applied with stress treatments and then moved to RM-IK17SF (osmotic medium with Ficoll-400) / PCL2-ppmsl (EDM)
CL = Cold treatment (control)
C = Centrifugation at 168g for 10 min.
E = Electroporation using 3 pulses of 200 V, 25 Ω and 25 µF
O = Osmotic shock

Table 4. Effect of stress treatments on anther induction, embryo formation and plant regeneration on CDC Luna

Treatments	% Anthers showing induction	Number of Embryos Produced	No. of Embryos that Formed Plants (p) Shoots (s) Roots (r)	No. of plants that survived in soil
Cold control	39	51	0	-
Osmotic shock	36	29	11(r)	-
Centrifugation + osmotic	27	30	8(s)	-
Electroporation + osmotic	36	24	2(p)	0
Centrifugation+Electroporation+Osmotic	34	12	3(p)	1(Fig. 10B)

3.2.3. Cultivar CDC Frontier

Table 5 presents the summary of all the treatments and media used on cultivar CDC Frontier. This cultivar was also bred at the Crop Development Centre, University of Saskatchewan, Saskatoon and it belongs to the kabuli class. Three base types of osmotic medium (JC-9, RM-9 and RM-IK-17) were tested in combination with various embryo development media. This cultivar was less responsive to the different treatments compared to cultivars Bumper and CDC Luna. Anther induction frequency ranged from 0 to 27% and embryo induction from 0 to 17 embryos (Table 5). The maximum anther induction rate of 27% was obtained in this cultivar when anthers were subjected to centrifugation, electroporation and osmotic shock (CEO) treatment using RM-IK17 (Appendix 1) and embryo development medium D8 (Appendix 2). A maximum of 17 embryos were produced from anthers subjected to the CEO treatment, the osmotic medium RM-IK17 (Appendix 1) and the embryo development medium PCL2-ppmss (Appendix 2, Table 5). This number is considerably lower than other cultivars indicating that further improvements are necessary.

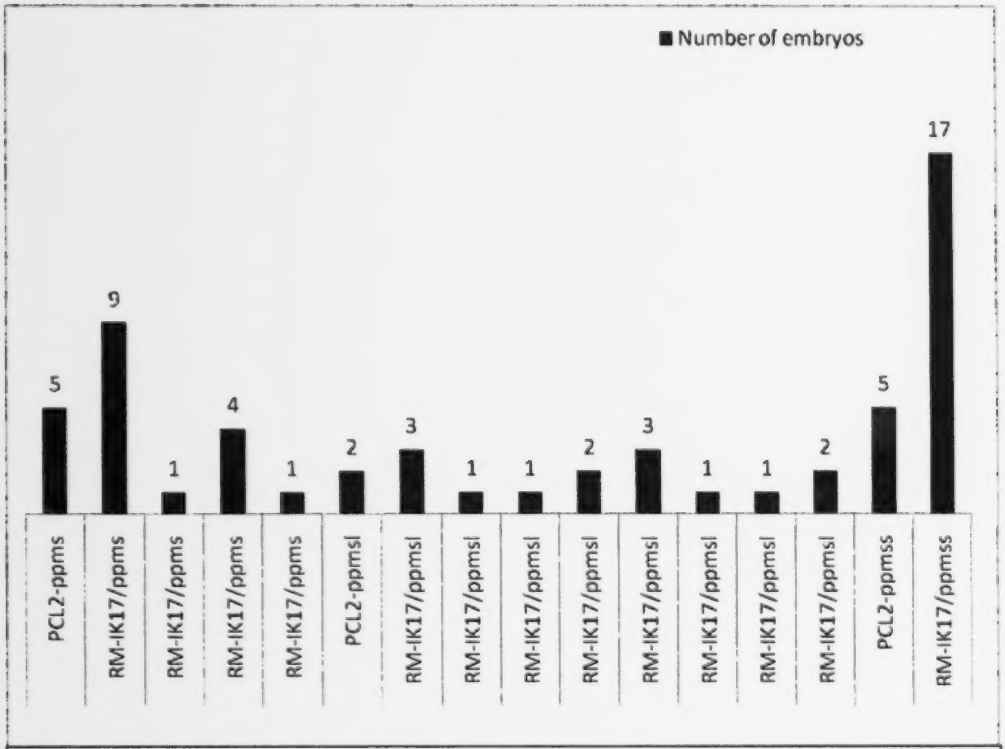
Table 5. Effect of different stress treatments, liquid and solid medium on anther and embryo induction for cultivar CDC Frontier

Treatments				High Osmotic Medium	Embryo Development Medium	Incubation Temp. (° C)	Hours of Incuba-tion	% Anther Induction	Number of Embryos
CL	C	E	O						
+	-	-	-	-	PCL2-pps			4	1
+	-	-	-	-	PCL2-ppms			5	5
+	-	-	-	-	PCL2-ppmss			6	5

+	-	-	-	-	PCL2-ppmsl			21	2
+	-	-	+	JC9	PCL2-ppms			3	1
+	+	-	+	JC9	PCL2-ppms			6	1
+	-	+	+	JC9	PCL2-ppms			3	1
+	+	+	+	JC9	PCL2-ppms			3	1
+	-	-	+	RM-9	PCL2-ppms			8	2
+	+	-	+	RM-9	PCL2-ppms			10	2
+	-	+	+	RM-9	PCL2-ppms			2	1
+	+	+	+	RM-9	PCL2-ppms			11	8
+	-	-	+	RM-IK17	PCL2-ppms			7	9
+	-	-	+	RM-IK17	PCL2-ppmsA			15	4
+	+	-	+	RM-IK17	PCL2-ppms			7	1
+	-	+	+	RM-IK17	PCL2-ppms			14	4
+	+	+	+	RM-IK17	PCL2-ppms			6	1
+	-	-	+	RM-IK pH5.9	PCL2-pps			17	7
+	-	-	+	RM-IK pH7.0	PCL2-pps			4	1
+	+	-	+	RM-IK17	PCL2-pps			11	1
+	-	+	+	RM-IK17	PCL2-pps			17	6
+	+	+	+	RM-IK17	PCL2-pps			17	4
+	-	-	+	RM-IK17	PCL2-ppms-1			8	1
+	-	-	+	RM-IK17	D4			3	0
+	-	-	+	RM-IK17	D5			16	3
+	-	-	+	RM-IK17	D5	32.5	16	17	0
+	-	-	+	RM-IK17	D7			8	1
+	-	+	+	RM-IK17	D7			17	1
+	+	+	+	RM-IK17	D7			0	0
+	-	-	+	RM-IK17	D8			18	1
+	-	-	+	RM-IK17	D8	32.5	16	19	1
+	+	-	+	RM-IK17	D8			16	2
+	-	+	+	RM-IK17	D8			20	1
+	+	+	+	RM-IK17	D8			27	2
+	-	-	+	RM-IK17	PCL2-ppmsl			5	3
+	+	-	+	RM-IK17	PCL2-ppmsl			4	1
+	-	+	+	RM-IK17	PCL2-ppmsl			6	1

Legend:
CL = Cold treated flower clusters
C = Centrifugation using 168 g for 10 min.
E = Electroporation with 3 pulses using 200 V, 25 Ω and 25 µF
O = Osmotic shock for 4 days prior to transfer to embryo development medium
LSD = Least significant difference at 0.05

Fig.14. Effect of stress treatments and embryo development media on embryo production in CDC Frontier

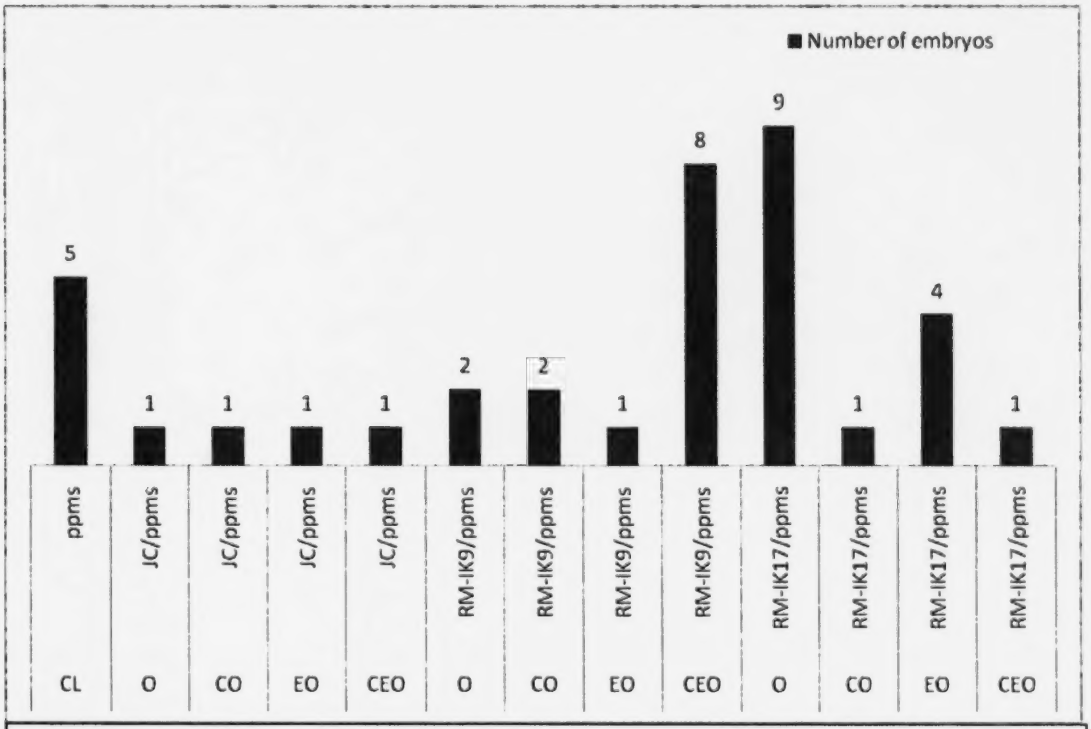


Legend:
ppms = PCL2-ppms (EDM)
ppmss = PCL2-ppmss (EDM)
ppmsl = PCL2-ppmsl (EDM)
RM-IK17 / ppms = RM-IK17 (osmotic medium) / PCL2-ppms (EDM)
RM-IK17 / ppmss = RM-IK17 (osmotic medium) / PCL2-ppmss (EDM)
RM-IK17 / ppmsl = RM-IK17 (osmotic medium) / PCL2-ppmsl (EDM)
CL = Cold treatment (control)
C = Centrifugation at 168g for 10 min.
E = Electroporation using 3 pulses of 200 V, 25 Ω and 25 µF
O = Osmotic shock

Fig.14 represents embryo production of anthers cultured on different embryo development media after application of all stress treatments using RM-IK17 as osmotic medium (Appendix 1). Anthers from cold treated flower clusters cultured on PCL2-ppms, PCL2-ppmsl, or PCL2-ppmss served as control. A maximum production of 17 embryos

was achieved in anthers subjected to the CEO treatments using the RM-IK17 and PCL2-ppmss medium combination. This treatment was followed by 9 embryos produced on anthers subjected to osmotic shock (O) treatment using the RM-IK17 and PCL2-ppms medium combination (Fig.14). Control (cold) anthers cultured on the same embryo development medium or on PCL2-ppmss produced 5 embryos. The difference between the treatments was significant at P> 0.05 (Table 5).

Fig. 15. Effect of three osmotic media and stress treatments on embryo production of CDC Frontier



Legend:
ppms = PCL2-ppms (EDM)
JC/ppms = JC (osmotic medium) / PCL2-ppms (EDM)
RM-IK9/ppms = RM-IK9 (osmotic medium) / PCL2-ppms (EDM)
RM-IK17/ppms = RM-IK17 (osmotic medium) / PCL2-ppms (EDM)
CL = Cold treatment (control)
C = Centrifugation at 168g for 10 min.
E = Electroporation using 3 pulses of 200 V, 25 Ω and 25 µF
O = Osmotic shock

The effect of three different osmotic media, namely JC, RM-IK9 and RM-IK17, was evaluated for embryo production using the different stress treatments (Fig. 15). These anthers were then cultured on PCL2-ppms (Appendix 2). Results of this study showed

that osmotic shock (O) treatment using RM-IK17/ppms medium was the best treatment producing 9 embryos compared to the CEO + RM-IK9/ppms treatment which produced 8 embryos (Fig. 15). The frequency of embryo formation in the cold control was 5 embryos. The difference in embryo production rates between both treatments and the control was significant at the P> 0.05 (Table 5).

Table 6. Effect of stress treatments on anther induction, embryo formation and plant regeneration of CDC Frontier

Treatments	% Anthers Showing Induction	Number of embryos	No. of Embryos that Formed Plants (p) Shoots (s) Roots (r)	No. of plants that survived in soil
Cold control	9	3	0	
Osmotic shock	11	2	4(r)	
Centrifugation + osmotic	9	1	2(s)	
Electroporation+osmotic	11	2	1(p)	1 (Fig.8C)
Centrifugation+Electroporation+ Osmotic	12	5	1(p) 3(r)	

Table 6 summarizes anther and embryo induction as well as regeneration for CDC Frontier. Embryos obtained from this cultivar were first transferred to maturation medium EMM-1 (Appendix 3) and then to JR1 medium in Magenta® boxes (Appendix 4) for regeneration. Although the frequency of anther and embryo induction in this cultivar was low compared to Bumper and CDC Luna, two plantlets were recovered. The plantlet obtained via the C+E+O treatment had few short roots but did not survive transfer to soil. The other plantlet from the E+O treatment was moved to a filter-paper bridge in a test tube and liquid medium with rooting hormones was added. After 5 days this plantlet formed a well developed root system and survived transfer to soil (Photo file Fig. 8C). It seems that this cultivar might need higher voltages of electrical current to produce stronger root systems. A contributing negative factor was also the low light intensity in our growth chamber necessitated by infrastructure problems in the phytotron. Low light intensity causes poor growth and low hormone levels in plants.

3.2.4. Cultivar Narayen

Australian cultivar Narayen belongs to the kabuli class. Similar to CDC Frontier, this cultivar was also less responsive in terms of frequency of embryo formation. Anther induction ranged from 0 to 18% and embryo production varied from 0 to 20 embryos (Table 7). Maximum anther induction of 18% was achieved in two treatments. First, anthers were exposed to osmotic shock (O) for four days in RM-IK17 and kept at 32.5° C for 16 hours and second, anthers were kept in RM-IK2F (medium with Ficoll-400; Appendix 1) prior to transfer to PCL2-ppms medium for embryo development. An anther induction rate of 17% was also obtained by the osmotic shock (O) treatment in RM-IK17

for four days prior to transfer to D3 (Appendix 2) indicating that Narayen preferred osmotic shock treatment for anther induction (Table 7).

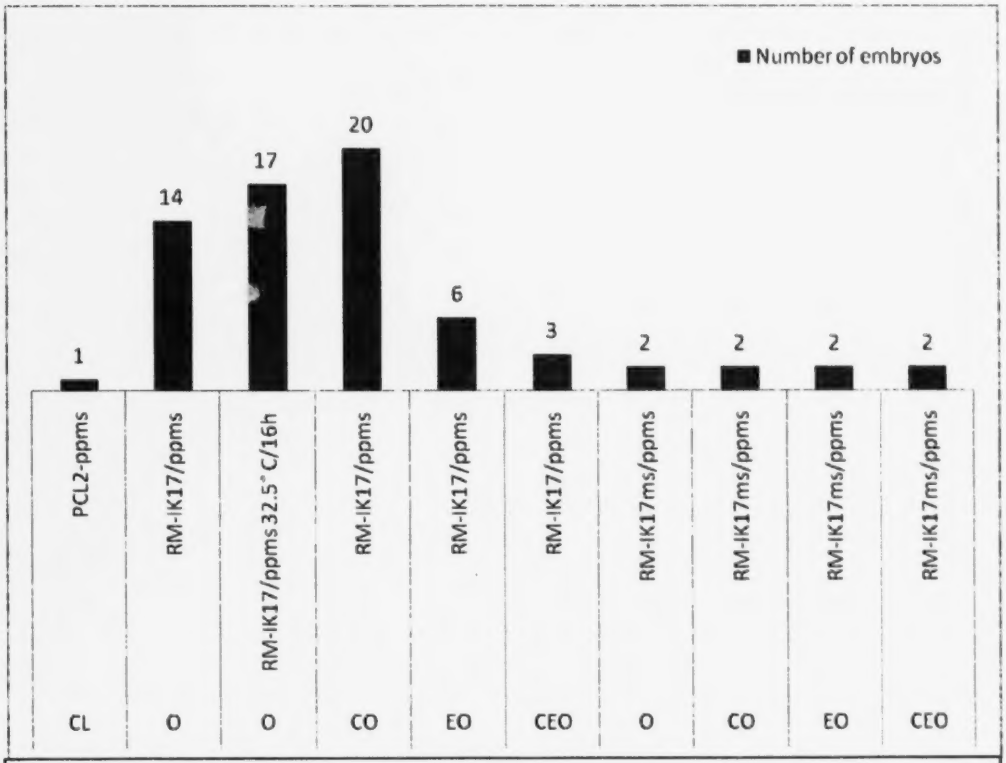
Table 7. Effect of different stress treatments, liquid and solid medium on anther and embryo induction for cultivar Narayen

Treatment				Osmotic Medium	Embryo Development Medium	Incubation Temp. °C	Hours of Incu-bation	% Anther Induction	Number of Embryos
CL	C	E	O						
+	-	-	-	-	PCL2-ppms			1	1
+	-	-	-	-	PCL2-ppmss			7	7
+	-	-	-	-	PCL2-ppmsl			10	1
+	-	-	+	RM-IK17ms	PCL2-ppms			7	2
+	+	-	+	RM-IK17ms	PCL2-ppms			3	2
+	-	+	+	RM-IK17ms	PCL2-ppms			7	2
+	+	+	+	RM-IK17ms	PCL2-ppms			5	2
+	-	-	+	RM-IK17	PCL2-ppms			13	14
+	**+**	**-**	**+**	**RM-IK17**	**PCL2-ppms**			**8**	**20**
+	-	+	+	RM-IK17	PCL2-ppms			8	6
+	+	+	+	RM-IK17	PCL2-ppms			10	3
+	-	-	+	RM-IK17	PCL2-ppms	32.5	16	18	17
+	-	-	+	RM-IK17	PCL2-ppmss	32.5	16	3	1
+	-	-	+	RM-IK17	D3	32.5	16	17	0
+	-	-	+	RM-IK2 to RM-IK-2F	PCL2-ppmss			7	1
+	+	+	+	RM-IK2 to RM-IK-2F	PCL2-ppmss			13	3
+	+	+	+	RM-IK2 to RM-IK-2F	PCL2-ppms			10	1
+	-	-	+	RM-IK-2F	PCL2-ppmss			18	3
+	-	-	+	RM-IK-2F	PCL2-ppmsl			5	0
+	-	+	+	RM-IK-2F	PCL2-ppmsl			12	2
+	+	+	+	RM-IK-2F	PCL2-ppmsl			12	4
+	-	-	+	RM-IK-2F	PCL2-ppms			6	3
+	-	-	+	RM-IK17	PCL2-ppmss			2	1
+	-	+	+	RM-IK17	PCL2-ppmss			0	0
+	+	-	+	RM-IK17	PCL2-ppms			16	3
+	-	-	+	RM-IK17SF	PCL2-ppmsl			13	5
+	+	+	+	RM-IK17SF	PCL2-ppmsl			12	1
+	-	-	+	RM-IK17SF	PCL2-ppms			10	4
+	+	+	+	RM-IK17SF	PCL2-ppms			6	1
+	-	-	+	RM-IK17	PCL2-ppmsl			7	1
+	-	+	+	RM-IK17	PCL2-ppmsl			11	1
+	+	-	+	RM-IK17	PCL2-ppmsl			3	1
+	+	+	+	RM-IK17	PCL2-ppmsl			3	9
+	-	-	+	RM-IK25	PCL2-ppmsl			6	1
+	-	+	+	RM-IK25	PCL2-ppmsl			1	0
+	+	-	+	RM-IK25	PCL2-ppmsl			9	2
+	+	-	+	PCL2-ppmSP	PCL2-ppmss			12	11
+	-	+	+	PCL2-ppmSP	PCL2-ppmss			8	9
+	+	+	+	PCL2-ppmSP	PCL2-ppmss			15	4
						LSD 0.05 =		**4.2**	**3.3**

Legend:
CL = Cold treated flower clusters
C = Centrifugation at 168 g for 10 min.
E = Electroporation using 200 V, 25 Ω and 25 µF; 3 pulses
O = Osmotic shock medium for 4 days prior to transfer to embryo development medium
LSD = Least significant difference at 0.05

In contrast, Narayen preferred the combination of centrifugation and osmotic shock (CO) treatment when it comes to embryo production (20 embryos), followed by osmotic shock (O) treatment alone which produced 17 embryos (Table 7). Figure 16 shows the 20 embryos were produced from anthers of the centrifugation and osmotic (CO) shock treatment for four days in RM-IK17 prior to the transfer to PCL2-ppms medium. If anthers using the same medium combination were either incubated at 32.5° C for 16 hours or continuously incubated at 24° C during the osmotic shock (O) treatment, then 17 and 14 embryos were produced, respectively (Fig. 16). The difference between these treatments was not significant at P> 0.05 (Table 7).

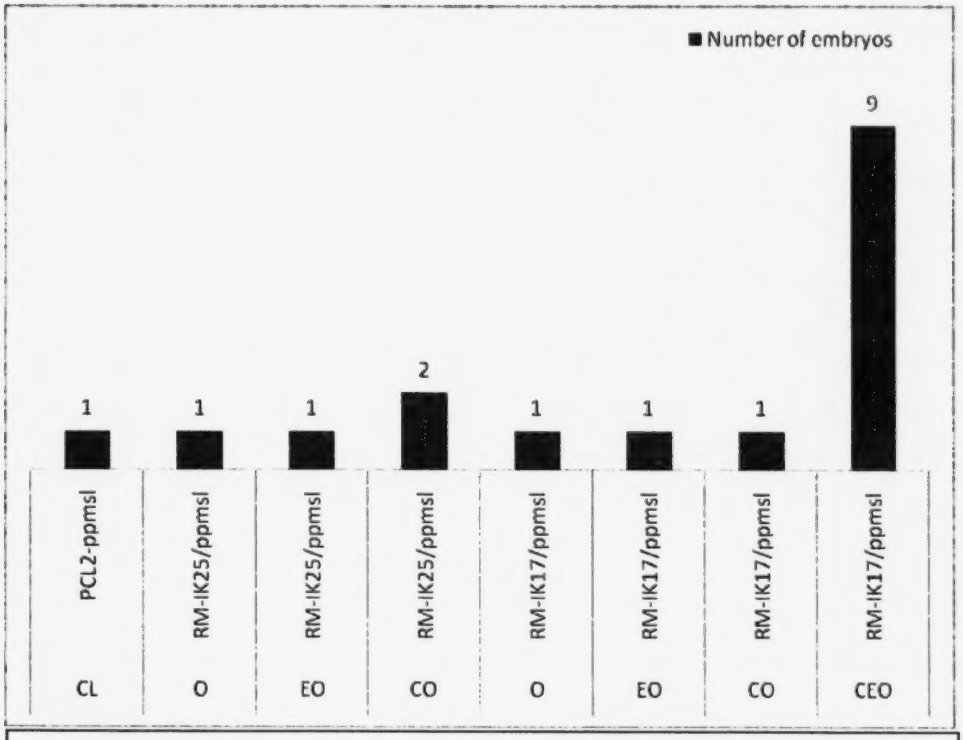
Fig.16. Effect of stress treatments, osmotic and embryo development media on embryo production of Narayen



Legend:
PCL2-ppms = PCL2-ppms (EDM)
RM-IK17/ppms = RM-IK17 (Osmotic medium) / PCL2-ppms (EDM)
RM-IK17ms/ppms = RM-IK17ms (Osmotic medium) / PCL2-ppms (EDM)
RM-IK17/ppms 32.5°C/16 = Anthers induced in RM-IK17 (Osmotic medium), incubated at 32.5°C for 16 h and then transferred to ppms (PCL2-ppms, EDM)
CL = Cold treatment (control)
C = Centrifugation at 168g for 10 min.
E = Electroporation using 3 pulses of 200 V, 25 Ω and 25 μF
O = Osmotic shock

Comparison of two osmotic media, RM-IK25 and RM-IK17 (Appendix 1), was performed keeping embryo development medium (PCL2-ppmsl) the same for all the treatments. Results of this study showed that anthers in RM-IK17 during the CEO treatment and subsequently moved to PCL2-ppmsl medium produced 9 embryos, whereas all other treatments produced only 1 or 2 embryos (Fig. 17). These results were significantly different at P> 0.05 (Table 7).

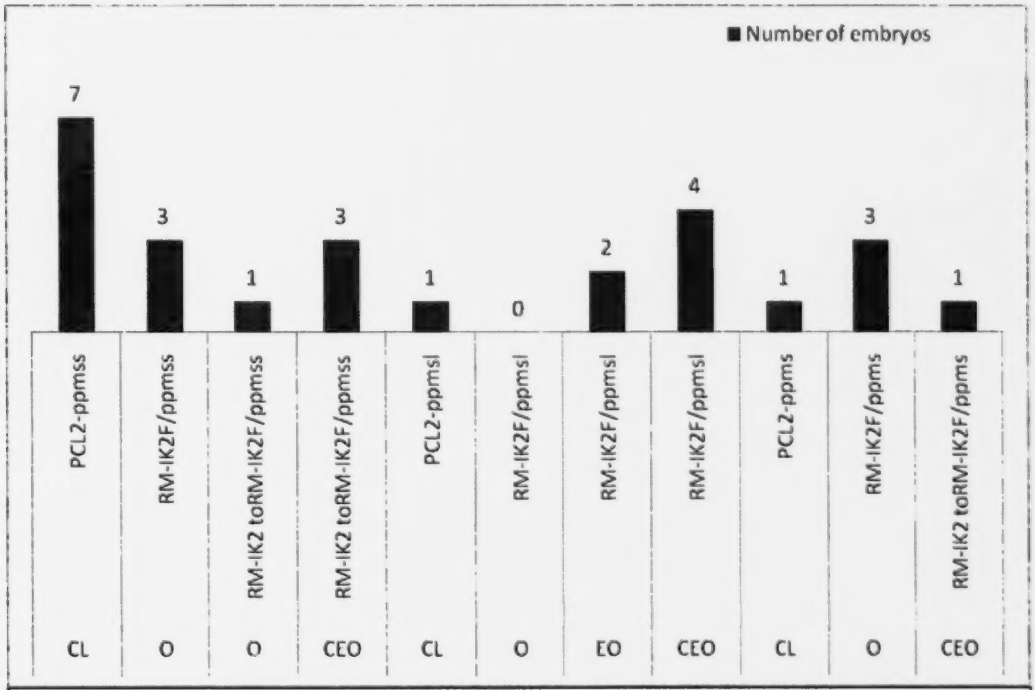
Fig.17. Effect of stress treatments, osmotic media and Larcoll-containing EDM medium (PCL2-ppmsl) on embryo production on Narayen



Legend:
PCL2-ppmsl = EDM with Larcoll
RM-IK25/ppmsl = RM-IK25 (osmotic medium) / PCL2-ppmsl (EDM with Larcoll)
RM-IK17/ppmsl = RM-IK17 (osmotic medium) / PCL2-ppmsl (EDM with Larcoll)
CL = Cold treatment (control)
C = Centrifugation at 168g for 10 min.
E = Electroporation using 3 pulses of 200 V, 25 Ω and 25 µF
O = Osmotic shock

Similar to the other cultivars, Narayen anthers were also treated with the osmotic medium containing 300 g/l Ficoll-400 (RM-IK2F; Appendix 1) in order to observe its impact on frequency of embryo formation. Anthers were either incubated directly in the Ficoll-400 containing medium for all the stress treatments or the centrifugation and electroporation treatments were given in medium without Ficoll-400 and then the anthers moved to Ficoll-400 containing medium. Results showed that the addition of Ficoll did not improve the frequency of embryo formation compared to the control. Control anthers produced 7 embryos, whereas Ficoll-treated anthers produced 4, 3, 2 or 1 embryo (Fig. 18). These differences were significant from control anthers at P> 0.05 (Table 7).

Fig.18. Effect of Ficoll-400, stress treatments and embryo development media on embryo production of cultivar Narayen



Legend:
RM-IK2F/ppmss = RM-IK2F (osmotic medium with Ficoll)/PCL2-ppmss (EDM with Larcoll)
RM-IK2 to RM-IK2F/ppmss = Anthers induced in RM-IK2 (osmotic medium without Ficoll) during stress treatments, then transferred to RM-IK2F (medium with Ficoll) during 4-day high osmotic treatment followed by transfer to PCL2-ppmss (EDM)
RM-IK2F/ppmsl = RM-IK2F (osmotic medium) / PCL2-ppmsl (EDM with Larcoll)
RM-IK2F/ppms = RM-IK2F (osmotic medium) / PCL2-ppms (EDM)
RM-IK2 to RM-IK2F/ppms = Anthers induced in RM-IK2 (osmotic medium without Ficoll) during stress treatments, then moved to RM-IK2F (osmotic medium with Ficoll) during 4-day high osmotic treatment followed by transfer to PCL2-ppms (EDM)
CL = Cold treatment (control)
C = Centrifugation at 168g for 10 min.
E = Electroporation using 3 pulses of 200 V, 25 Ω and 25 µF
O = Osmotic shock

Similar to the other cultivars, the embryos obtained in this experiment (Fig. 7D) were moved to embryo maturation medium (EMM-3A) and then transferred to regeneration medium. The results obtained from cultivar Narayen are presented in Table 8. Since this cultivar was screened only recently, data on plantlet regeneration (Fig. 8D) and survival into soil have not yet been evaluated (Table 8).

Table 8 Effect of stress treatments on anther induction, embryo formation and plant regeneration of Narayen

Treatments	% Anthers Showing Induction	Number of Embryos	No. of Embryos that Formed Plants (p) Shoots (s) Roots (r)
Cold control	6	3	0
Osmotic shock	9	3	1(r)
Centrifugation + osmotic	9	7	0
Electroporation + osmotic	7	3	I(s) 5(r)
Centrifugation+Electroporation+Osmotic	8	3	1(p) (Fig. 8D)

3.2.5. ICRISAT breeding line ICCV2

Regarding the ICRISAT breeding line ICCV2, four different osmotic media (JC, RM-IK9, RM-IK17 and BKHS) were tested (Appendix 1). For all the different stress treatments, embryo development medium PCL2-ppms or CIM-ppms (Appendix 2) was used. In control (cold) treatment, anthers were cultured on embryo development medium that was sterilized by autoclaving or by using 0.2 µm bottle top filters. ICCV2 belongs to the desi class and was the genotype that was the least responsive to our DH protocol.

Table 5 presents the summary of experiments performed for this cultivar. The maximum anther induction rate of 37% (Table 9) was achieved when anthers were submitted to centrifugation and osmotic shock (CO) treatment in BKHS (Appendix 1) for four days prior to the transfer to PCL2-ppms medium. However, this did not translate into embryo production. The highest number of 18 embryos was achieved by anthers that were given centrifugation, electroporation and osmotic shock (CEO) treatment using the RM-IK17 and PCL2-ppms medium combination (Table 9). Embryo formation occurred normally (Fig. 7E), but they could not be regenerated. All embryos from ICCV2 became green and then later on died (Fig. 9C).

Table 9. Effect of different stress treatments, liquid and solid medium on anther and embryo induction for cultivar ICCV2

Treatment				Osmotic Medium	Embryo Development Medium	% Anthers Induction	Number of Embryos
CL	C	E	O				
+	-	-	-	-	PCL2-ppms	12	5
+	-	-	-	-	PCL2-ppmsA	22	3
+	-	-	-	-	CIM-ppms	25	2
+	-	-	+	JC	PCL2-ppms	24	3
+	+	-	+	JC	PCL2-ppms	12	3
+	-	+	+	JC	PCL2-ppms	25	1
+	+	+	+	JC	PCL2-ppms	17	4
+	-	-	+	RM-IK9	PCL2-ppms	12	2
+	+	-	+	RM-IK9	PCL2-ppms	12	1
+	-	+	+	RM-IK9	PCL2-ppms	11	1
+	+	+	+	RM-IK9	PCL2-ppms	**29**	**2**
+	-	-	+	RM-IK17	PCL2-ppms	16	2
+	+	-	+	RM-IK17	PCL2-ppms	19	3
+	-	+	+	RM-IK17	PCL2-ppms	22	3
+	**+**	**+**	**+**	**RM-IK17**	**PCL2-ppms**	**31**	**18**
+	-	-	+	BKHS	PCL2-ppms	13	0
+	**+**	**-**	**+**	**BKHS**	**PCL2-ppms**	**37**	**0**
+	-	+	+	BKHS	PCL2-ppms	25	0
+	+	+	+	BKHS	PCL2-ppms	10	0
					LSD 0.05 =	**10.8**	**3.8**

Legend:
CL = Cold treated flower clusters (control)
C = Centrifugation using 168 g for 10 min.
E = Electroporation using 3 pulses with 200 V, 25 Ω and 25 µF
O = Osmotic shock of anthers in medium for 4 days prior to transfer to EDM
LSD = Least significant difference at 0.05

3.3. F1 hybrids

Any efficient double haploid protocol must be applicable to F1 hybrids from a wide range of crosses. Therefore, hybrids arising from 10 different cross combinations were included towards the end of this project. To date, only data on embryo production is available on these hybrids with the exception of 21085MM9X618. These hybrids were 1936as, 1936bs, 1939bs, 1946bs, 1949s, 1990T, 2011T, 2014T, 2025T and 2180MM9X618. Five to eight

seeds from each hybrid were provided by Dr. Buyanmin Tar'an (Chickpea Breeder, The Crop Development Centre, University of Saskatchewan, Saskatoon). Testing of the regeneration protocol is still in progress.
For anther and embryo induction, the following stress treatments were applied:

- Cold treatment of all flower clusters (CL) = control
- Cold treatment of flower clusters followed by culturing anthers in high osmotic shock medium for 4 days prior to transfer to solid medium (O)
- Cold treatment of flower clusters followed by centrifugation of anthers using 168g for 10 min and by osmotic shock treatment for 4 days prior to the transfer to solid medium (CO)
- Cold treatment of flower clusters followed by electroporation of anthers using 200 V, 25 Ω and 25 μF and osmotic shock treatment for 4 days prior to the transfer to solid medium (EO)
- Cold treatment of flower clusters followed by centrifugal, electrical and osmotic shock of anthers (CEO) prior to the transfer to solid medium

RM-IK17 generally used as osmoticum during the centrifugation (C), electroporation (E) and 4-dα, high osmotic (O) treatments (Appendix 1.) Anthers were then transferred to solid embryo development medium PCL2-ppms unless otherwise stated (Appendix 2). Depending on the availability of donor material, some hybrid anthers were also transferred to PCL2-ppmss or PCL2-ppmsl medium (Appendix 2).

3.3.1. Hybrid 1936as

Hybrids were grown in the summer and fall of 2009. During this time, growth conditions were sub-optimal due to infrastructure problems in the phytotron. Plants did not receive full light intensity which is required for high anther and embryo induction response.
Anthers from hybrid 1936as were subjected to all the stress treatments using RM-IK17 and cultured either on embryo development medium PCL2-ppms or PCL2-ppmss (Table 10). Maximum anther induction of 35% but only 7 embryos were obtained in the cold control (CL). Nine embryos were produced when anthers were given the osmotic shock (O) treatment for 4 days and then cultured on PCL2-ppms; eight embryos were produced in the CEO treatment, where anthers were moved to PCL2-ppmsl medium (Table 10 These differences were not significant (P> 0.05).

3.2.2. Hybrid 1936bs

Table 10 describes the results obtained with hybrid 1936bs. Maximum anther induction of 33% and 8 embryos was achieved in the control anthers (CL). Any other stress treatment did not improve anther induction rates or embryo production. Results were significant at P> 0.05 (Table 10).

Table 10. Effect of different stress treatments, liquid and solid medium on anther and embryo induction for hybrids 1936as, 1936bs, 1939bs and 1946bs

	Treatment				Osmotic	Embryo	%	Number
Legend: CL = Cold treated flower clusters C = Centrifugation stress treatment at 168 g for 10 min. E = Electroporation using 3 pulses and 200 V, 25 Ω and 25 µF O = Osmotic shock, keeping anthers in high osmotic pressure medium for 4 days prior to transfer to embryo development medium LSD = Least significant difference at 0.05								
1936as	+	+	+	+	RM-IK17	PCL2-ppms	6	2
1936as	+	+	+	+	RM-IK17	PCL2-ppmss	8	1
1936as	+	+	+	+	RM-IK17	PCL2-ppmsl	18	8
						LSD 0.05=	**7.6**	**3.8**
1936bs	+	-	-	-	-	PCL2-ppms	33	8
1936bs	+	+	-	+	RM-IK17	PCL2-ppms	22	1
1936bs	+	-	+	+	RM-IK17	PCL2-ppms	18	3
1936bs	+	+	+	+	RM-IK17	PCL2-ppms	20	4
1936bs	+	+	+	+	RM-IK17	PCL2-ppmsl	5	3
						LSD 0.05=	**5.5**	**3.3**
1939bs	+	-	-	-	-	PCL2-ppms	39	5
1939bs	+	+	-	+	RM-IK17	PCL2-ppms	43	6
1939bs	+	-	+	+	RM-IK17	PCL2-ppms	23	2
1939bs	+	+	+	+	RM-IK17	PCL2-ppms	30	7
1939bs	+	+	+	+	RM-IK17	PCL2-ppms	19	5
						LSD 0.05=	**15**	**7.6**
1946bs	+	-	-	-	-	PCL2-ppms	27	4
1946bs	+	-	-	+	RM-IK17	PCL2-ppms	7	1
1946bs	+	-	-	+	RM-IK17	PCL2-ppmsl	3	1
1946bs	+	+	-	+	RM-IK17	PCL2-ppms	20	3
1946bs	+	-	+	+	RM-IK17	PCL2-ppms	9	2
1946bs	+	+	+	+	RM-IK17	PCL2-ppms	14	3
1946bs	+	+	+	+	RM-IK17	PCL2-ppmsl	11	1
						LSD 0.05=	**8.8**	**4.4**

3.3.3. Hybrid 1939bs

In hybrid 1939bs, the maximum anther induction rate of 43% was observed in the electroporation and osmotic shock (CO) treatment (Table 10). When anthers were cultured on PCL2-ppms medium, 6 embryos were produced. Embryo production in this hybrid ranged from 2 to 7 embryos, the latter obtained from the CEO treatment. The difference among all the treatments in terms of embryo production was not significant at P> 0.05 (Table 10).

3.3.4 Hybrid 1946bs

Hybrid 1946bs showed a maximum anther induction rate of 27% and of 4 embryos from the cold treatment (CL) (Table 10) similar to the hybrid 1936bs. All subsequent treatments did not improve induction.

3.3.5. Hybrid 1949bs

In hybrid 1949bs anther induction frequency ranged from 6 to 27%, whereas embryo production varied from 2 to 17 embryos (Table 11). Maximum anther induction of 27% was achieved from the cold treatment, followed by 19% in the osmotic shock (O) treatment. Regarding embryo production, the combination of centrifugation and osmotic shock (CO) treatment produced 17 embryos (Fig. 19A). Although this treatment did not differ significantly from the control, it differed significantly from all other stress treatments at P> 0.05 (Table 11).

3.3.6. Hybrid 1990T

The results of all the stress treatments applied to the anthers of this hybrids are presented in Table 15. Although this hybrid did not show good response to DH protocol, but among all the treatments given to the anthers, CC was the best in terms of anther and embryo induction. There is no data available on regeneration, needed to be evaluated.

3.3.7 Hybrid 2011T

In hybrid 2011T, only 3 treatments were tested due to the scarcity of plant material. Hybrid 2011T also showed the best results in terms of anther and embryo induction with only cold (CL) treatment (Table 11). This hybrid produced an anther induction rate of 26%

and 5 embryos (Fig. 19B). Differences among these observations for both anther and embryo induction were not significant at P> 0.05.

Table 11. Effect of different stress treatments, liquid and solid medium on anther and embryo induction for hybrids 1949bs, 1990T, 2011T, 2014T and 2025T

Hybrid	Treatment				Osmotic Medium	Embryo Development Medium	% Anther induction	Number of embryos
	CL	C	E	O				
1949bs	+	-	-	-	-	PCL2-ppms	27	10
1949bs	+	-	-	+	RM-IK17	PCL2-ppms	19	2
1949bs	**+**	**+**	**-**	**+**	**RM-IK17**	**PCL2-ppms**	**11**	**17 Fig. 19A**
1949bs	+	-	+	+	RM-IK17	PCL2-ppms	6	3
1949bs	+	+	+	+	RM-IK17	PCL2-ppms	11	3
						LSD 0.05	**9.6**	**9.9**
1990T	+	-	-	-	RM-IK17	PCL2-ppms	19	3
1990T	+	-	-	+	RM-IK17	PCL2-ppms	10	1
1990T	+	+	-	+	RM-IK17	PCL2-ppms	9	1
1990T	+	-	+	+	RM-IK17	PCL2-ppms	7	1
1990T	+	+	+	+	RM-IK17	PCL2-ppms	2	0
1990T	+	+	+	+	RM-IK17	PCL2-ppmsl	10	1
						LSD 0.05	**9.1**	**1.8**
2011T	**+**	**-**	**-**	**-**	**RM-IK17**	**PCL2-ppms**	**26**	**5 Fig 19B**
2011T	+	+	-	+	RM-IK17	PCL2-ppms	8	1
2011T	+	+	+	+	RM-IK17	PCL2-ppms	8	3
						LSD 0.05	**25.6**	**7.9**
2014T	+	-	-	-	RM-IK17	PCL2-ppms	36	7
2014T	+	-	-	+	RM-IK17	PCL2-ppms	42	6
2014T	+	+	-	+	RM-IK17	PCL2-ppms	15	4
2014T	+	-	+	+	RM-IK17	PCL2-ppms	31	4
2014T	+	+	+	+	RM-IK17	PCL2-ppms	20	5
						LSD 0.05	**25.9**	**5.2**
2025T	+	-	-	-	RM-IK17	PCL2-ppms	43	8
2025T	+	-	-	+	RM-IK17	PCL2-ppms	19	4
2025T	+	+	-	+	RM-IK17	PCL2-ppms	23	1
2025T	+	-	+	+	RM-IK17	PCL2-ppms	30	8
2025T	+	+	+	+	RM-IK17	PCL2-ppms	24	5
						LSD 0.05	14.9	3.0

Legend:
CL = Cold treated flower clusters
C = Centrifugation using 168 g for 10 min.
E = Electroporation using 3 pulses and 200 V, 25 Ω and 25 µF
O = Osmotic shock, keeping anthers in high osmotic pressure medium for 4 days prior to transfer to embryo development medium
LSD = Least significant difference at 0.05

3.3.8. Hybrid 2014T

Table 17 shows the frequency of anther and embryo induction in hybrid 2014T. The anther induction frequency ranged from 15 to 42% with maximum anther induction obtained in the osmotic (O) treatment (Table 17). Frequency of embryo formation ranged from 4 to 7 embryos but differences among all treatments in terms of embryo production were not significant at P> 0.05.

3.3.9 Hybrid 2025T

Like most of the hybrids, hybrid 2025T also showed maximum anther induction frequency of 43% in the cold (CL) treatment (Table 11). Frequency of embryo formation ranged from 1 to 8 embryos. Both the cold (CL) and the electroporation plus osmotic shock (EO) treatments produced 8 embryos.

3.3.10. Hybrid 21080MM9X618

Maximum anther induction of 26% occurred in the osmotic shock treatment (Table 12). Only 2 embryos were produced from the CEO treatment. Interestingly, this hybrid produced one plantlet to date from this treatment (Fig. 9A). This plantlet is still on PRM and will be transferred to soil in the near future.

Table 12. Effect of different stress treatments, liquid and solid medium on anther and embryo induction for hybrid 21080mm9x618

Treatment	Osmotic	Embryo	%	Number of
Legend: CL = Cold treated flower clusters C = Centrifugation using 168 g for 10 min. E = Electroporation using 3 pulses and 200 V, 25 Ω and 25 µF O = Osmotic shock, keeping anthers in high osmotic pressure medium for 4 days prior to transfer to embryo development medium LSD = Least significant difference at 0.05				
		LSD 0.05 =	**5.6**	**1.9**

3.4. Ploidy level

The ploidy level of callus, embryos and plants at different growth stages was determined using flow cytometry and chromosome counts, as presented in Fig. 20 (Photo file), profiles a-d. The profiles clearly show peaks corresponding to the C (at an intensity emitted of epifluorescence below 100) and the 2C (at around 200 to 250 intensity of emitted epifluorescence) relative DNA nuclear content. In this respect, the DNA profiles observed from embryos and callus during early growth phases revealed haploid profiles (Photo file Figs. 20b, c, e & f), whereas regenerated plants of Bumper (Photo file Figs. 20a - c) and CDC Luna (Photo file Figs. 20d - f) obtained from cold flower cluster treatment followed by anther electroporation showed spontaneous doubling of chromosomes during early regeneration stages.

4. Summary

The developed DH protocol was tested on five cultivars and ten hybrids.

1. Haploid embryos were produced from all cultivars, breeding lines and hybrids tested.
2. Plants were produced from cultivars Bumper, CDC Luna, CDC Frontier, Narayen and hybrid 21085MM9X618.
3. Anther electroporation also enhanced root formation which is a major hurdle in many legume regeneration protocols.
4. Addition of Ficoll-400 to the osmotic medium or pH manipulation of the same medium did not improve regeneration frequency in any cultivar.
5. Modifications to the osmotic shock and embryo development medium increased embryo induction rates, but did not improve plant regeneration.
6. These results may have been influenced by the less than optimal donor plant growth due to infrastructure problems in the phytotron facility. Donor plants were grown under sub-optimal conditions with considerably lower light intensity and shorter photoperiod than required for optimal plant growth.
7. Overall, the developed DH protocol is suitable for a wide variety of cultivars, breeding lines and hybrids.
8. Regeneration frequency is still low and hence, this protocol needs further improvements to be used routinely in chickpea breeding.

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C. Personnel

Dr. Ravinder Grewal worked full time, whereas Dr. Monika Lulsdorf worked part time on this project. Ali Shaterian was hired as at technician in May 2008 and left this project in December 2008. Shermy Mudiyanselage was hired as a technician in February 2009.

D. Equipment

No equipment was purchased with this grant.

E. Project Developed Materials

Publication:

Grewal R., Lulsdorf M., Croser J., Ochatt S., Vandenberg A and Warkentin T. D. (2008) Doubled Haploid Production in Chickpea (*Cicer arietinum* L) – Role of Stress Treatments Plant Cell Reports 28: 1289 – 1299

Publication in preparation:

Grewal R., Lulsdorf M., Croser J., Ochatt S., Vandenberg A and Warkentin T. D. (2010) Response of diverse chickpea accessions to doubled haploid anther culture protocol Plant Cell Tiss Organ Cult

Oral Presentations:

Grewal R., Lulsdorf M., Croser J., Warkentin T and Vandenberg A. (2008) Progress towards androgenesis from intact anther culture in chickpea (*Cicer arietinum* L.) Presented at the 7th Canadian Tissue Culture Workshop in Saskatoon May 5th – 8th, 2008

Grewal R., Lulsdorf M., Croser J., Ochatt S., Vandenberg A and Warkentin T.D. (2008) Double Haploid Production in Chickpea and Field Pea. Presented at the 7th Canadian Pulse Research Workshop in Winnipeg, MB Nov. 4th – 7th, 2008

Grewal R (2009) Double haploid technology for chickpea and field pea presented PLSC 990 seminar, Department of Plant Sciences, University of Saskatchewan, Saskatoon, September 2009.

Poster Presentation:

Grewal R., Lulsdorf M., Croser J., Warkentin T and Vandenberg A. (2008)
Stress for Success: Towards Haploid Chickpea Production. Poster presented at Pulse Days in Saskatoon on Jan. 10-11, 2008.

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Screening of chickpea cultivars, breeding lines and hybrids

F. Project Photos

Photos are attached in a separate file.

G. Acknowledgments

Support of the Saskatchewan Agriculture Development Fund was acknowledged in all presentations in which this research was described.

H. Expense statement

Will be provided separately.

Appendix 1 Osmotic Media

Components mg/L	BKHS	JC	JC15	RM-IK9	RM-IK17
KCl	1491.00				
KNO_3		2500.00	2500.00	61.00	61.00
NH_4NO_3					
$MgSO_4 \cdot 7H_2O$	246.50	370.00	370.00	150.00	150.00
KH_2PO_4	136.10	170.00	170.00	67.00	67.00
Na_2EDTA		37.30	37.30	37.30	37.30
$FeSO_4 \cdot 7H_2O$		27.80	27.80	27.80	27.80
$CaCl_2 \cdot 2H_2O$		600.00	600.00		
$Ca(NO_3)_2 \cdot 4H_2O$				500.00	500.00
$MnSO_4 \cdot H2O$		22.30	22.30	20.00	20.00
H_3BO_3		6.20	6.20	10.00	10.00
$ZnSO_4 \cdot 7H_2O$		10.00	10.00	15.00	15.00
$Na_2MoO_4 \cdot 2H_2O$		0.25	0.25	0.25	0.25
$CuSO_4 \cdot 5H_2O$		0.05	0.05	0.05	0.05
$CoCl_2 \cdot 6H_2O$		0.025	0.025	0.05	0.05
KI		0.83	0.83	1.00	1.00
Thiamine HCl		0.50	0.50	0.10	0.10
Nicotinic acid		5.00	5.00	1.00	1.00
Pyridoxine HCl		0.50	0.50	0.50	0.50
Myo-inositol		100.00	100.00	1000.00	1000.00
L-Glutamine				800.00	800.00
Casein hydrolysate		200.00	200.00		
Folic acid				1.00	1.00
D+ Biotin				0.10	0.10
Glycine		2.00	2.00	2.00	2.00
Cholecalciferol (vitamin D)				0.10	0.10
Cyanocobalamin (vitamin B_{12})				0.01	0.01
Riboflavin				0.05	0.05
Sorbitol (%)	2.73				
Sucrose (%)	2.73	9.00	15.00	3.00	17.00
Maltose (%)				6.00	
Picloram		0.30	0.30		
2,4-D		0.05	0.05		
BAP		0.08	0.08		
IAA				4.00	4.00
Kinetin				0.40	0.40
Calcium pantothenate				1.00	1.00
pH	**6.0**	**5.8**	**5.8**	**6.60**	**6.60**

Components mg/L	RM-IK-1	RM-IK2NF	RM-IK2F	RM-IK-pH5.9	RM-IK-pH7.0	RM-IK 17SP	RM-IK 17SF
KNO_3	61.00	61.00	61.00	61.00	61.00	61.00	61.00
$MgSO_4 \cdot 7H_2O$	150.00	150.00	150.00	150.00	150.00	150.00	150.00
KH_2PO_4	67.00	67.00	67.00	67.00	67.00	67.00	67.00
Na_2EDTA	37.30	37.30	37.30	37.30	37.30	37.30	37.30
$FeSO_4 \cdot 7H_2O$	27.80	27.80	27.80	27.80	27.80	27.80	27.80
$Ca(NO_3)_2 \cdot 4H_2O$	500.00	250.00	250.00	500.00	500.00	500.00	500.00
$MnSO_4 \cdot H_2O$	20.00	20.00	20.00	20.00	20.00	20.00	20.00
H_3BO_3	10.00	5.00	5.00	10.00	10.00	10.00	10.00
$ZnSO_4 \cdot 7H_2O$	15.00	15.00	15.00	15.00	15.00	15.00	15.00
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	0.25	0.25	0.25	0.25	0.25
$CuSO_4 \cdot 5H_2O$	0.25	0.025	0.025	0.25	0.25	0.50	0.50
$CoCl_2 \cdot 6H_2O$	0.25	0.025	0.025	0.25	0.25	0.50	0.50
KI	0.83	0.83	0.83	0.83	0.83	1.00	1.00
Thiamine HCl	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Nicotinic acid	0.50			0.50	0.50	1.00	1.00
Pyridoxine HCl	1.00	1.00	1.00	1.00	1.00	0.50	0.50
Myo-inositol	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00
L-Glutamine	500.00	500.00	500.00	500.00	500.00	800.00	800.00
Casein hydrolysate	100.00	100.00	100.00	100.00	100.00		
Folic acid	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D+ Biotin	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Glycine	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Cholecalciferol (vitamin D)	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Cyanocobalamin (vitamin B_{12})	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Riboflavin	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Ficoll-400 (g)			300.00				300.00
Glutathione (Red.)	50.00	50.00	50.00	50.00	50.00		
Sucrose (%)	9.00	9.00	9.00	9.00	9.00	7.00	7.00
Maltose (%)				3.00	3.00	10.00	10.00
Mannitol (%)				2.00	2.00		
Lactose (%)				2.00	2.00		
Pyruvic acid				89.00	89.00		
Serine	100.00	100.00	100.00	100.00	100.00		
Larcoll	15.00	15.00	15.00	15.00	15.00		
2,4-D	0.05	0.05	0.05	0.05	0.05		
Picloram						1.00	1.00
ABA	1.00	1.00	1.00	1.00	1.00		
IAA	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Kinetin	0.400	0.400	0.400	0.400	0.40	0.40	0.40
Calcium pantothenate	1.00	1.00	1.00	1.00	1.00	1.00	1.00
pH	**6.60**	**6.60**	**6.60**	**5.9**	**7.0**	**6.60**	**6.60**

Components mg/ L	RM-IK-17b	HSO-P	HSO-P3	PCL2-ppmsP	ChM-GM
KNO_3	61.00	61.00	61.00	2100.00	2500.00
NH_4NO_3				1000.00	
$MgSO_4 \cdot 7H_2O$	250.00	250.00	250.00	435.00	370.00
KH_2PO_4	67.00	61.00	61.00	325.00	170.00
Na_2EDTA	37.30	37.30	37.30		
FeSO4 $\cdot$7H2O	27.80	27.80	27.80		
$CaCl_2 \cdot 2H_2O$			370.00	600.00	600.00
$Ca(NO_3)_2 \cdot 4H_2O$	150.00	500.00	500.00		
NaH_2PO_4				85.00	
FeNaEDTA				25.00	27.85
$MnSO_4 \cdot H_2O$	20.00	18.95	18.95	15.00	16.91
H_3BO_3	10.00	10.00	10.00	5.00	6.20
$ZnSO_4 \cdot 7H_2O$	15.00	8.60	8.60	5.00	10.00
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	0.25	0.4	0.25
$CuSO_4 \cdot 5H_2O$	0.50	0.025	0.025	0.1	0.05
$CoCl_2 \cdot 6H_2O$	0.50	0.025	0.025	0.1	0.025
KI	1.00	0.83	0.83	1.00	0.83
Thiamine HCl	0.10	0.10	0.10	2.00	0.50
Nicotinic acid	0.50				5.00
Pyridoxine HCl	1.00	0.5	0.5	0.50	0.50
Myo-inositol	1000.00	500.00	500.00	250.00	100.00
L-Glutamine	800.00	800.00	800.00		
Casein hydrolysate		250.00	250.00	300.00	200.00
Folic acid	1.00	1.00	1.00		
D+ Biotin	0.10	0.10	0.10	0.10	
Glycine	2.00	2.00	2.00		
Cholecalciferol (vitamin D)	0.10	0.2	0.2	0.10	
Cyanocobalamin (vitamin B12)		0.01	0.01	0.01	
Riboflavin	0.05			0.05	
Glutathione (Red.)			30.00	1.00	
Sucrose (%)	7.00	9.00	6.00	17.00	9.00
Maltose (%)	10.00		3.00		
BAP					0.10
2iP				0.40	
Serine	100.00	100.00	100.00		
Larcoll	15.00				
2,4-D	0.05				0.53
Picloram		1.00	1.00	1.00	0.25
ABA	1.00				
IAA	4.00				
Kinetin	0.400				
Calcium pantothenate	1.00			1.00	
pH	**6.60**	**5.8**	**5.8**	**5.8**	**5.8**

Appendix 2 Embryo Development Media (EDM)

Components mg/L	PCL2-pps	PCL2-ppms	PCL2-ppmsA	PCL2-ppmss	PCL2-ppmsi
KNO_3	2100.00	2100.00	2100.00	2100.00	2100.00
NH_4NO_3	1000.00	1000.00	1000.00	1000.00	1000.00
$MgSO_4 \cdot 7H_2O$	435.00	435.00	435.00	435.00	435.00
$CaCl_2 \cdot 2H_2O$	600.00	600.00	600.00	600.00	600.00
KH_2PO_4	325.00	325.00	325.00	325.00	325.00
NaH_2PO_4	85.00	85.00	85.00	85.00	85.00
NaFeEDTA	25.00	25.00	25.00	25.00	40.00
KI	1.00	1.00	1.00	0.83	2.00
H_3BO_3	5.00	5.00	5.00	5.00	5.00
$MnSO_4 \cdot H_2O$	15.00	15.00	15.00	15.00	15.00
$ZnSO_4 \cdot 7H_2O$	5.00	5.00	5.00	5.00	5.00
$Na_2MoO_4 \cdot 2H_2O$	0.40	0.40	0.40	0.40	0.40
$CuSO_4 \cdot 5H_2O$	0.10	0.10	0.10	0.10	0.10
$CoCl_2 \cdot 6H_2O$	0.10	0.10	0.10	0.10	0.10
Myo-Inositol	250.00	250.00	250.00	250.00	250.00
Pyridoxine HCL	0.50	0.50	0.50	0.50	0.50
Thiamine HCL	2.00	2.00	2.00	2.00	2.00
Glutathione (red.)					1.00
Casein Hydrolysate				300.00	300.00
Cholecalciferol (Vitamin D_3)				0.20	0.10
Cyanocobalamin (Vitamin B_{12})				0.01	0.01
Sucrose (%)	6.00	3.00	3.00	6.00	6.00
Maltose (%)		6.00	6.00	3.00	3.00
2,4-D				2.00	
2iP	0.40	0.40	0.40	0.40	0.40
Picloram	1.00	1.00	1.00	1.00	1.00
IAA					4.00
Larcoll					15.00
Serine				100.00	100.00
Agar (%)	1.60	1.60	1.60	1.60	1.60
pH	**5.8**	**5.8**	**5.8**	**5.8**	**5.8**

PCL2-ppmsA = Medium sterilization was done by autoclaving instead of filter sterilization used for all other media.

Components mg/L	PCL2-ppms-1	D1	D2	D3	D4
KNO_3	2100.00	2100.00	2100.00	2100.00	2100.00
NH_4NO_3	1000.00	1000.00	1000.00	1000.00	1000.00
$MgSO_4 \cdot 7H_2O$	435.00	435.00	435.00	435.00	435.00
$CaCl_2 \cdot 2H_2O$	600.00	600.00	600.00	600.00	600.00
KH_2PO_4	325.00	325.00	325.00	325.00	325.00
NaH_2PO_4	85.00	85.00	85.00	85.00	85.00
NaFeEDTA	25.00	25.00	25.00	25.00	25.00
KI	1.00	0.83	0.83	0.83	0.83
H_3BO_3	5.00	5.00	5.00	5.00	5.00
$MnSO_4 \cdot H_2O$	15.00	15.00	15.00	15.00	15.00
$ZnSO_4 \cdot 7H_2O$	5.00	5.00	5.00	5.00	5.00
$Na_2MoO_4 \cdot 2H_2O$	0.40	0.40	0.40	0.40	0.40
$CuSO_4 \cdot 5H_2O$	0.10	0.10	0.10	0.10	0.10
$CoCl_2 \cdot 6H_2O$	0.10	0.10	0.10	0.10	0.10
Myo-Inositol	250.00	250.00	250.00	250.00	250.00
Pyridoxine HCl	0.50	0.50	0.50	0.50	0.50
Thiamine HCl	2.00	2.00	2.00	2.00	2.00
Glutathione (red.)	10.00				
Casein Hydrolysate	300.00	300.00	300.00	300.00	300.00
Cholecalciferol (Vitamin D_3)	0.10	0.20	0.20	0.20	0.20
Cyanocobalamin (Vitamin B_{12})	0.01	0.01	0.01	0.01	0.01
Biotin	0.1				
Calcium pantothenate	1.00				
Sucrose (%)	4.50	6.00	6.00	6.00	6.00
Maltose (%)	4.50	3.00	3.00	3.00	3.00
2,4-D		0.50	1.00	2.00	10.00
2iP	0.40	0.40	0.40	0.40	0.40
Picloram	20.00	1.00	1.00	1.00	1.00
Serine		100.00	100.00	100.00	100.00
Agar (%)	1.60	1.60	1.60	1.60	1.60
pH	**5.8**	**5.8**	**5.8**	**5.8**	**5.8**

Components mg/L	D5	D6	D7	D8	CIM
KNO_3	2100.00	2100.00	2100.00	2100.00	1900.00
NH_4NO_3	1000.00	1000.00	1000.00	1000.00	1650.00
$MgSO_4 7H_2O$	435.00	435.00	435.00	435.00	370.00
$CaCl_2 \cdot 2H_2O$	600.00	600.00	600.00	600.00	440.00
KH_2PO_4	325.00	325.00	325.00	325.00	170.00
NaH_2PO_4	85.00	85.00	85.00	85.00	
NaFeEDTA	25.00	25.00	25.00	25.00	40.00
KI	0.83	0.83	0.83	0.83	0.83
H_3BO_3	5.00	5.00	5.00	5.00	6.20
$MnSO_4 \cdot H_2O$	15.00	15.00	15.00	15.00	22.30
$ZnSO_4 \cdot 7H_2O$	5.00	5.00	5.00	5.00	8.60
$Na_2MoO_4 \cdot 2H_2O$	0.40	0.40	0.40	0.40	0.25
$CuSO_4 \cdot 5H_2O$	0.10	0.10	0.10	0.10	0.025
$CoCl_2 \cdot 6H_2O$	0.10	0.10	0.10	0.10	0.025
Myo-Inositol	250.00	250.00	250.00	250.00	100.00
Pyridoxine HCl	0.50	0.50	0.50	0.50	1.00
Thiamine HCl	2.00	2.00	2.00	2.00	10.00
Nicotinic acid					1.00
Casein Hydrolysate	300.00	1000.00	300.00	300.00	
Cholecalciferol (Vitamin D_3)	0.20	0.20	0.20	0.20	
Cyanocobalamin (Vitamin B_{12})	0.01	0.01	0.01	0.01	
Sucrose (%)	6.00	9.00	6.00	6.00	3.00
Maltose (%)	3.00		3.00	3.00	6.00
2,4-D				2.00	0.25
2iP	0.40	0.40	0.40	0.40	0.40
Picloram	1.00	1.00	1.00	1.00	1.00
IAA			4.00	4.00	
Kinetin			0.40	0.40	0.025
BAP	0.10			0.10	
NAA	0.01			0.01	
DICAMBA		2.00			
Serine	100.00	100.00	100.00	100.00	
Agar (%)	1.60	1.60	1.60	1.60	1.60
pH	**5.8**	**5.8**	**5.8**	**5.8**	**5.8**

Appendix 3. Embryo Maturation Media (EMM)

Components mg/L	EMM-1	EMM-B	EMM-3A
KNO_3	2100.00	2100.00	2100.00
NH_4NO_3	1000.00	1000.00	1000.00
$MgSO_4 \cdot 7H_2O$	435.00	435.00	435.00
$CaCl_2 \cdot 2H_2O$	600.00	600.00	600.00
KH_2PO_4	325.00	325.00	325.00
NaH_2PO_4	85.00	85.00	85.00
NaFeEDTA	25.00	25.00	25.00
KI	0.80	0.83	0.80
H_3BO_3	5.00	5.00	5.00
$MnSO_4 \cdot H_2O$	15.00	15.00	15.00
$ZnSO_4 \cdot 7H_2O$	5.00	5.00	5.00
$Na_2MoO_4 \cdot 2H_2O$	0.40	0.40	0.40
$CuSO_4 \cdot 5H_2O$	0.10	0.10	0.10
$CoCl_2 \cdot 6H_2O$	0.10	0.10	0.10
Myo-Inositol	250.00	250.00	250.00
Pyridoxine HCl	0.50	0.50	0.50
Thiamine HCl	2.00	2.00	2.00
Glutamine	800.00		800.00
Glutathione (red.)	1.00		30.00
Casein Hydrolysate	300.00	300.00	300.00
Coconut water (ml)		20.00	
Cholecalciferol (Vitamin D_3)	0.20		0.20
Cyanocobalamin (Vitamin B_{12})	1.00		0.01
Biotin	0.10		
Calcium pantothenate	1.00		1.00
Sucrose (%)	3.00	4.50	6.00
Maltose (%)		4.50	
Serine		100.00	
GA_3	0.50	5.00	0.50
ABA		3.00	
Zeatin riboside		1.00	0.25
Picloram			1.00
IAA	2.00	4.00	2.00
Kinetin		0.40	
BAP	0.10		
NAA	0.01		
Larcoll	15.00		
Agar (%)	1.00	1.60	1.00
pH	**5.8**	**5.8**	**5.8**

Appendix 4. Plant Regeneration Media (PRM)

Components mg/L	PRM-JR1	PRM-JR1-(Rooting Hormone Medium)
KNO_3	1900.00	1900.00
NH_4NO_3	1650.00	1650.00
$MgSO_4 \cdot 7H_2O$	370.00	370.00
$CaCl_2 \cdot 2H_2O$	440.00	500.00
KH_2PO_4	170.00	200.00
Na_2 EDTA	37.30	37.30
$FeSO_4 \cdot 7H_2O$	27.80	27.80
KI	0.83	0.83
H_3BO_3	6.20	6.20
$MnSO_4 \cdot H_2O$	22.30	22.30
$ZnSO_4 \cdot 7H_2O$	8.60	8.60
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25
$CuSO_4 \cdot 5H_2O$	0.025	0.05
$CoCl_2 \cdot 6H_2O$	0.025	0.025
Myo-Inositol	100.00	100.00
Pyridoxine HCl	1.00	1.00
Thiamine HCl	10.00	10.00
Nicotinic acid	1.00	1.00
Glycine	0.025	0.025
Sucrose (%)	3.00	3.00
BAP	0.10	0.10
NAA	0.01	0.01
IBA		0.25
Cyanocobalamin (Vitamin B_{12})	0.01	0.01
Cholecalciferol (Vitamin D_3)	0.10	0.10
Calcium Pantothenate	1.00	1.00
Retinol	0.50	0.50
Casein Hydrolysate	0.10	0.10
Agar (%)	0.70	
pH	**5.8**	**5.8**

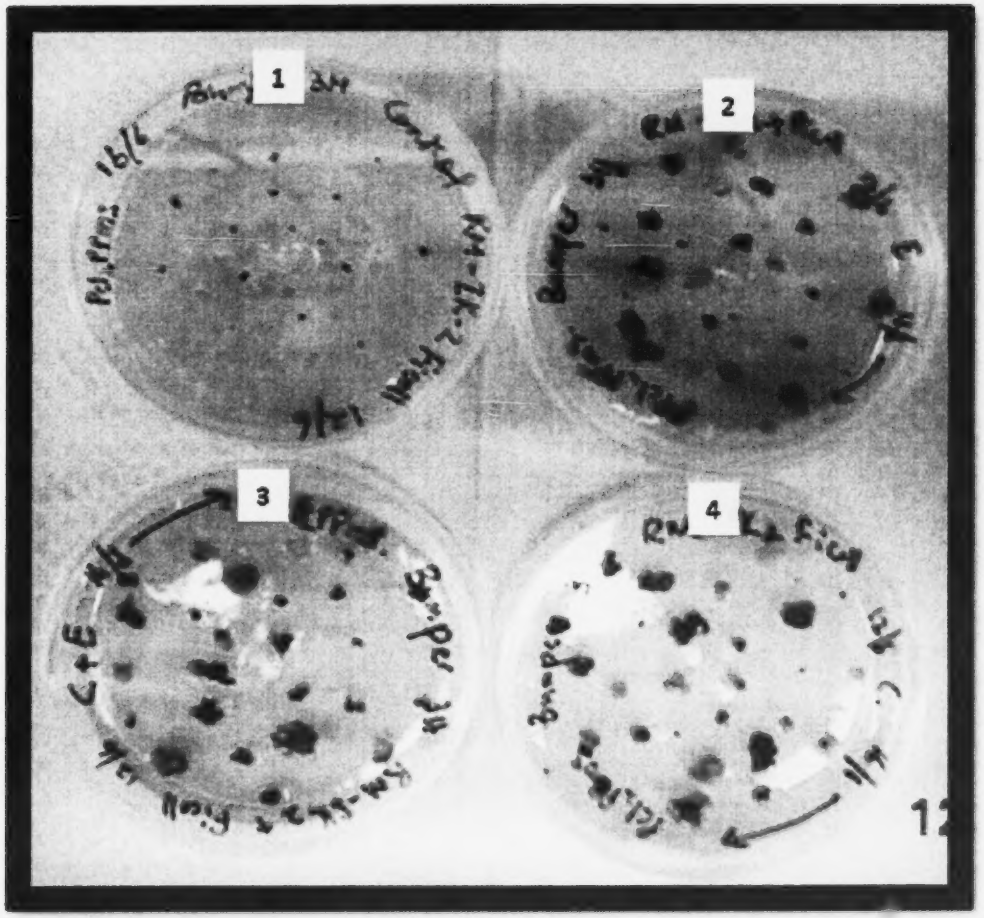


Fig. 6 Comparison of various treatments on anther and embryo induction of cultivar Bumper
1) Control anthers from cold treated flower clusters **2)** Anthers after electroporation with 3 exponentially decaying pulses using 200 V, 25 Ω and 25 µF **3)** Anthers centrifuged at 168 g for 10 minutes **4)** Combination of all treatments

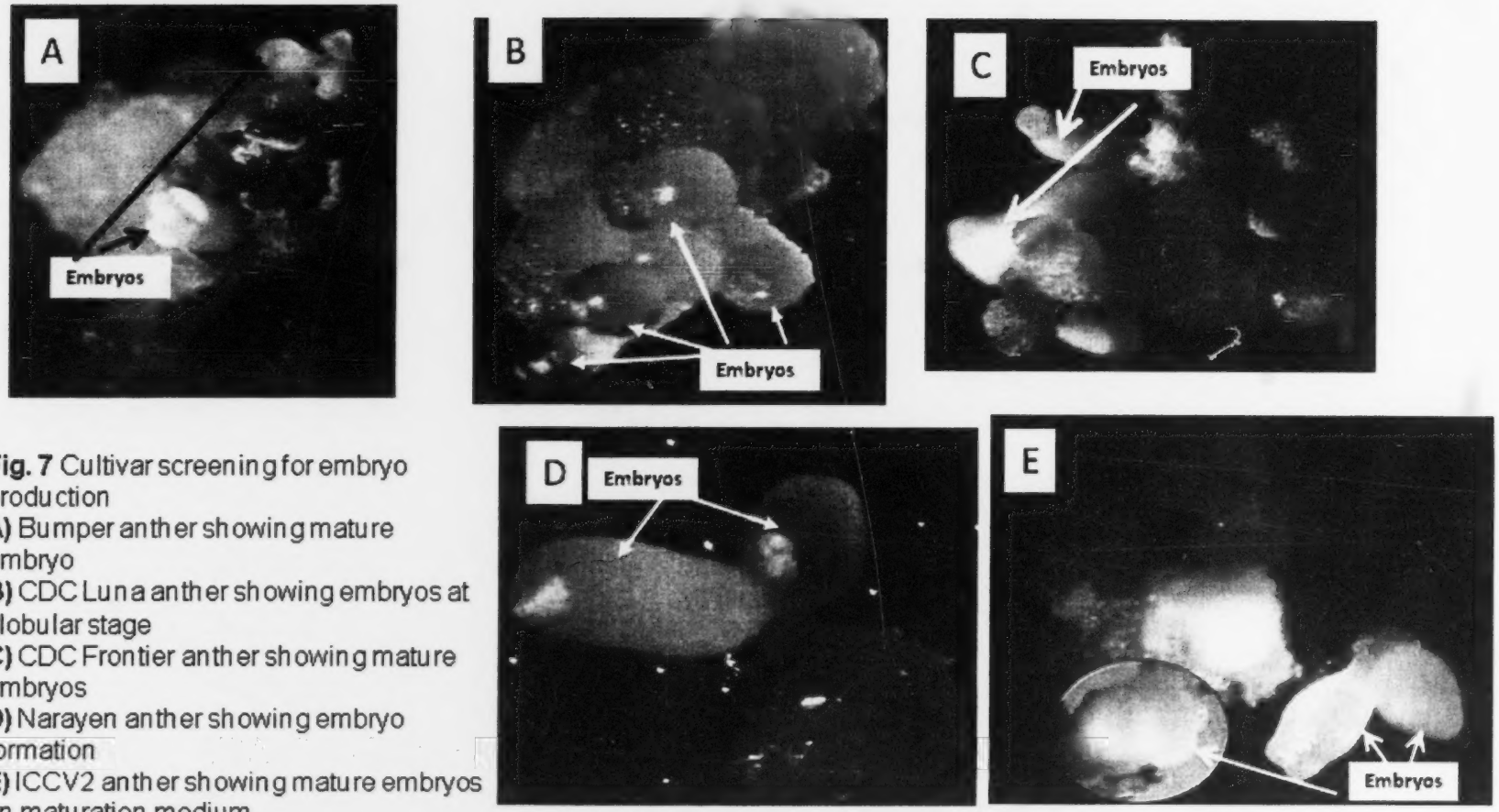


Fig. 7 Cultivar screening for embryo production
A) Bumper anther showing mature embryo
B) CDC Luna anther showing embryos at globular stage
C) CDC Frontier anther showing mature embryos
D) Narayen anther showing embryo formation
E) ICCV2 anther showing mature embryos on maturation medium

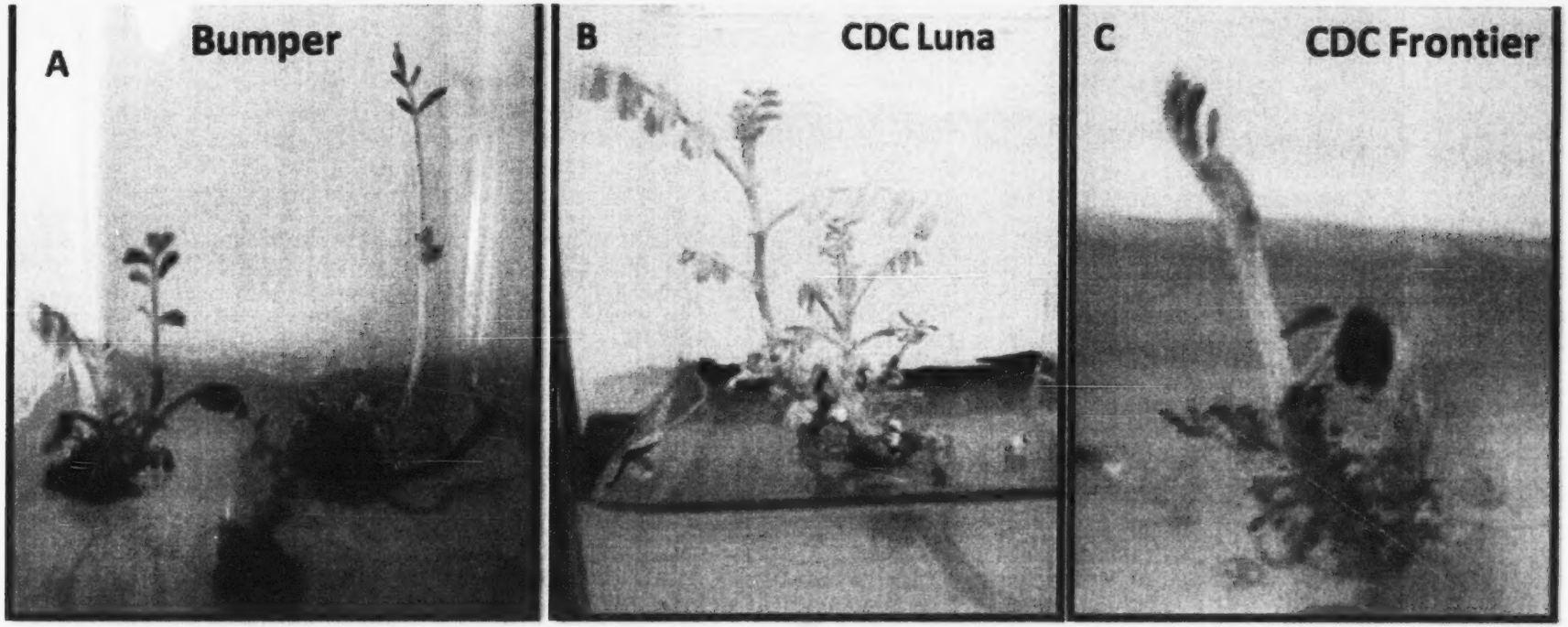
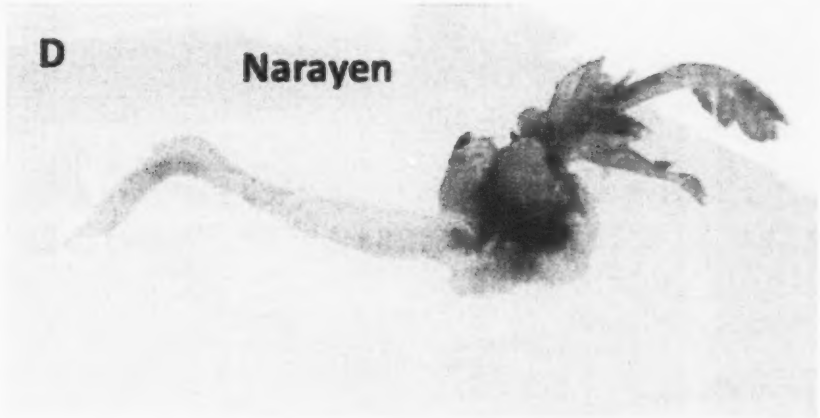


Fig. 8 Regeneration of screened cultivars
A) Plantlet formation from Bumper
B) Plantlet formation from CDC Luna
C) Plantlet formation from CDC Frontier
D) Plantlet formation from Narayen



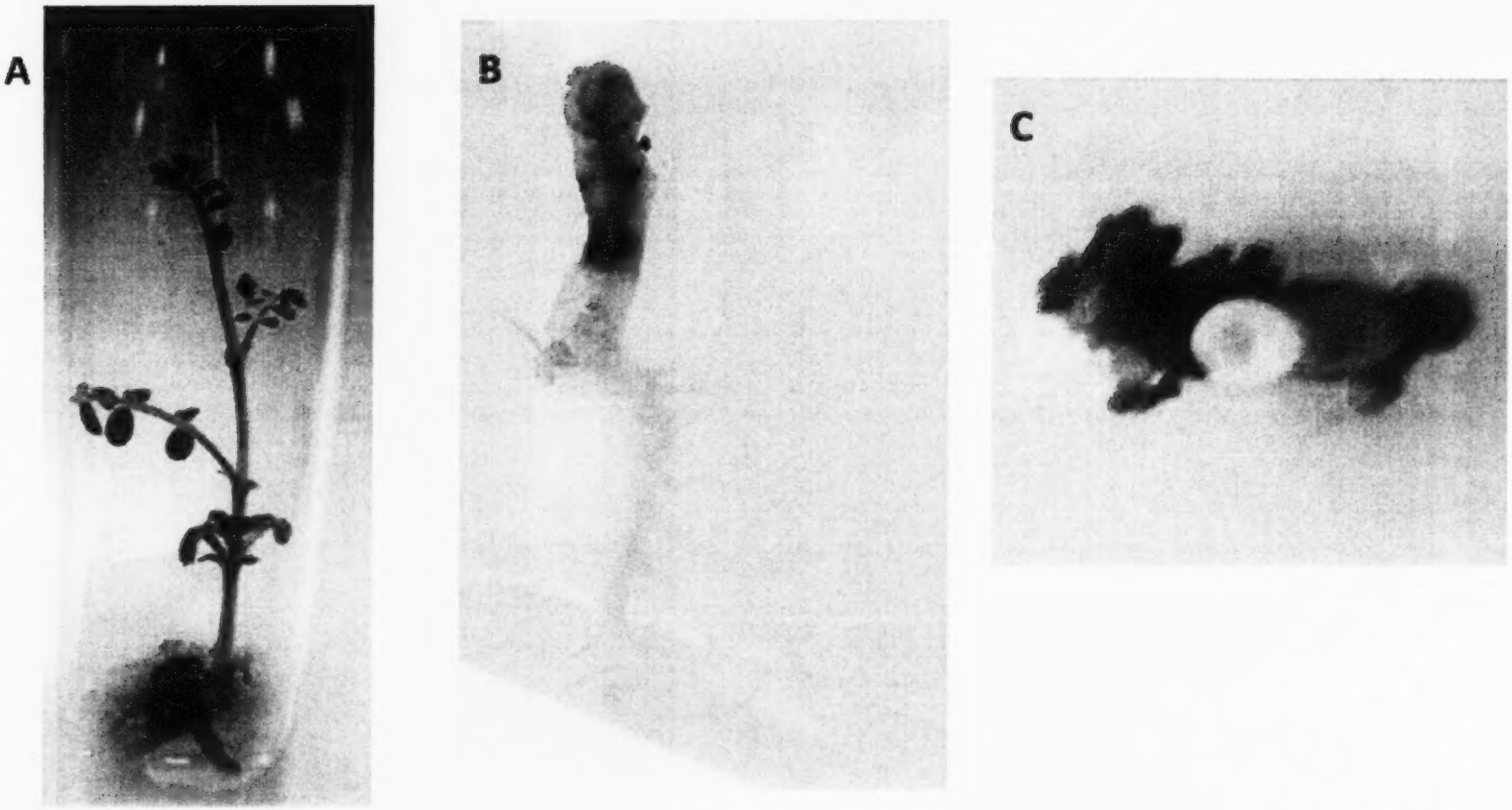


Fig. 9 Regeneration of different genotypes
A) Hybrid 21085MM9X618 showing plantlet formation
B) Bumper regeneration from CEO treatment using ChM-GM osmotic medium and embryo development medium PCL2-ppms PCL2- ppmss showing well developed roots but no shoot
C) ICCV2 embryo on plant regeneration medium did not show plant formation

Fig. 10 Pod formation from spontaneously doubled haploid plants under greenhouse conditions **A)** Bumper **B)** CDC Luna

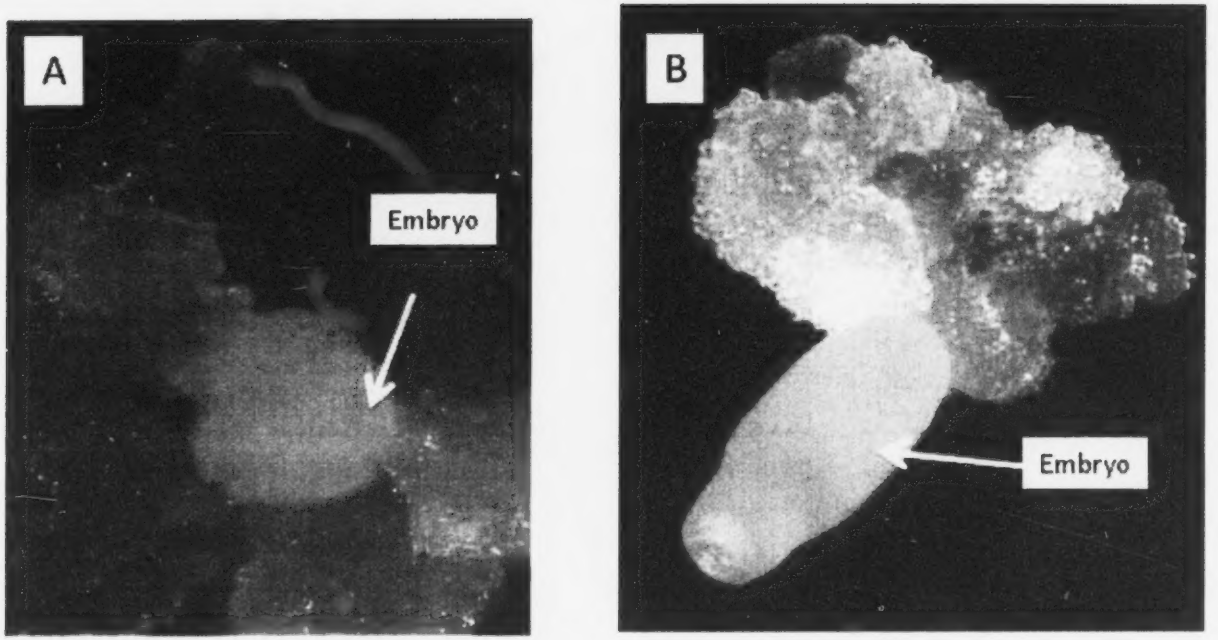


Fig.19 Embryo formation in hybrids
A) Hybrid 1949bs showing embryo formation on embryo development medium
B) Hybrid 2011T showing abnormal embryo on embryo development medium

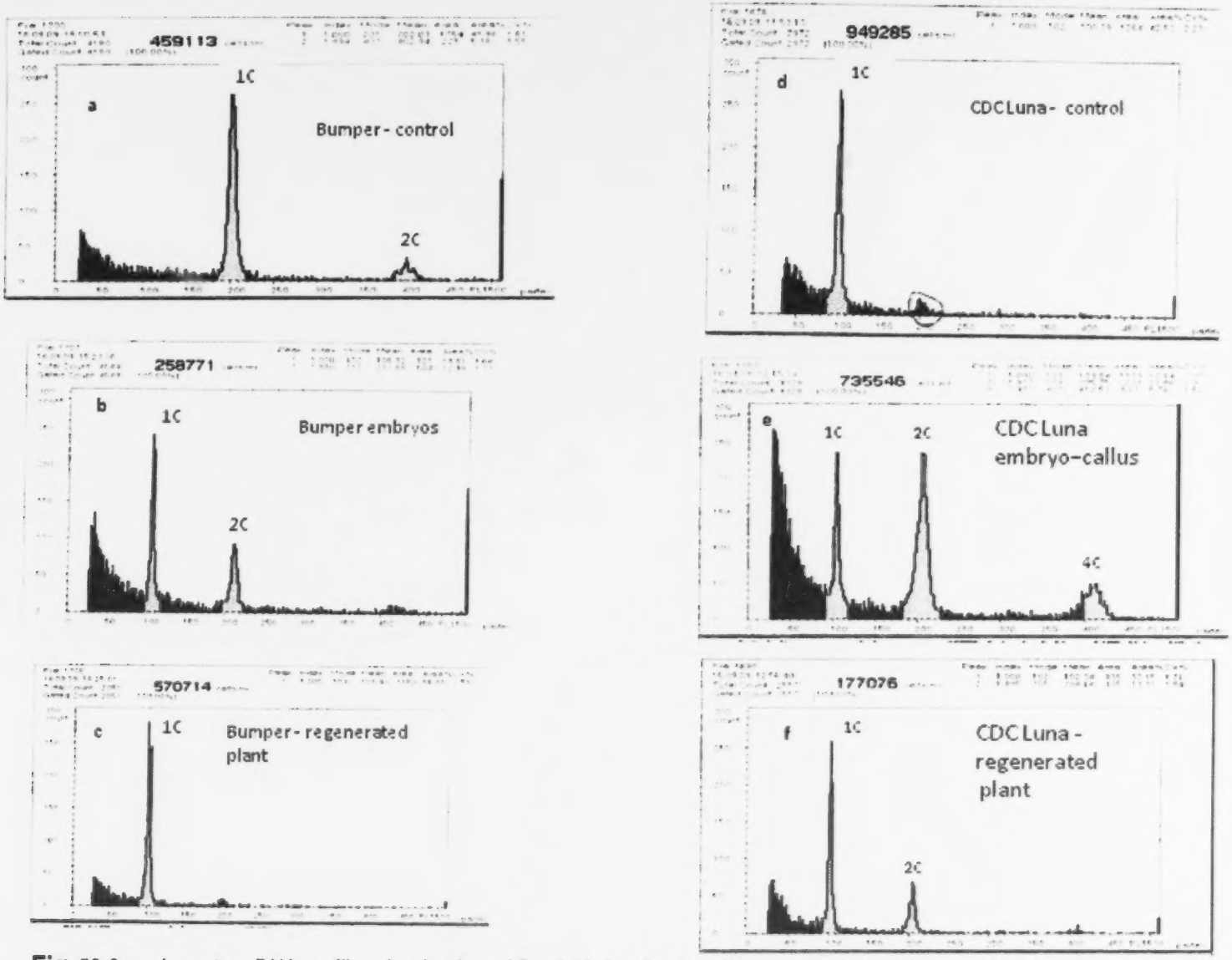


Fig. 20 Complementary DNA profiles showing 1c and 2c ploidy levels. **a)** Leaf from control plant of Bumper **b)** Embryos from combined treatment (CEO) of Bumper **c)** Leaf from regenerated plant of Bumper **d)** Leaf from control plant of CDC Luna **e)** Embryos and callus of CDC Luna **f)** Leaf from regenerated plant of CDC Luna